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Genetic modified pig production with combination of various gene modulating technologies

다양한 유전자 조절기술을 이용한 형질전환돼지의 생산

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Genetic modified pig production with combination of various gene modulating technologies

by Joon Ho Moon

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in

Theriogenology and Biotechnology
Department of Veterinary Medicine, Graduate School
Seoul National University

We accept this thesis as confirming to the required standard

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Seoul National University
December 2014

Declaration

This thesis is submitted by the undersigned for examination for the degree of Doctor of Philosophy to the Seoul National University. This thesis has not been submitted for the purposes of obtaining any other degree or qualification from any other academic institution.

I hereby declare that the composition and experiment of this thesis and the work presented in it are entirely my own.

Joon Ho Moon

Genetic modified pig production with combination of various gene modulating technologies

Joon Ho Moon

(Supervisor: Goo Jang, D.V.M., Ph.D.)

Theriogenology and Biotechnology

Department of Veterinary Medicine, Graduate School

Seoul National University

ABSTRACT

The most ideal way to produce cloned pigs to date is SCNT. Due to comparative simplicity of overexpression than gene KO, lots of target gene overexpressed pigs have been produced while less KO pigs were reported. Recently, diverse genetic scissors (i.e., ZFN, TALEN or RGEN), which rapidly increase the number of KO animals including pigs have been started to highlight.

Pigs are considered to be an important large animal model in biomedical research. Due to absence of porcine ESCs and characterized cell lines, development velocities of genetic modification technologies and transgenic pig production are tardy. In this study, to overcome absence of characterized cell lines, *hTERT* genes overexpressing immortalized cell lines were established and characterized. These cells were further used to apply genetic modification technology (i.e., KO by TALEN). The gene overexpression and KO technologies were respectively applied for genetic modified pig production (i.e., Neurodegenerative disorder pig model).

For immortalizing porcine cells, primary porcine fetal fibroblasts were isolated and cultured using the *hTERT* transfection. After selecting cells with neomycin for two weeks, outgrowing colonized cells were picked up and sub-cultured for expansion.

Various analyses were accomplished and characterized for confirming target gene insertion in cells. Immortalized cells were cultured for more than 9 months without changing their doubling time (approximately 24 h) or their diameter (< 20 μm) while control cells became senescent during the same period. Even a single cell expanded to confluence in 100 mm dishes.

Furthermore, to KO the *CMAH* gene, designed plasmids encoding a TALENs pairs were transfected into the immortalized cells. Each single colony was analyzed by the mutation sensitive T7E1 assay, fPCR and sequencing to obtain 3 independent clonal populations of cells that

contained biallelic modifications. The *GGTA1* gene KO processes was exactly same with that of *CMAH* KO cell line establishment method. One *hTERT* overexpressed plus *CMAH* KO clone was chosen and used for SCNT. Cloned embryos developed to the blastocyst stage.

To produce target gene overexpressed and knocked out pigs, PD related genes were selected; *SNCA* and *PRKN*.

Here, I produced 23 cloned pigs expressing human *SNCA via* SCNT. Among them, 4 pigs survived (designated PDF4, PDF16, PDF18 and PDF20) and were confirmed as transgenic animals by PCR based analysis. Target proteins were detected in only 2 transgenic pigs (PDF4 and PDF18) by ELISA at 4 months of age. However, SNCA concentrations in blood samples were changed at 1 year of age. PDF4 and PDF20 showed significantly increased levels of SNCA. The SNCA concentration in PDF18 significantly decreased, while that of PDF16 did not change during 1 year after birth. Behavior scoring analysis was carried out in control pigs and all cloned pigs at timed intervals (Control, unchanged; PDF18, decreased; PDF4, PDF16 and PDF20, increased; PD like behavioral changes lead score increasing). Similar changes were detected in both ELISA and behavior scoring analyses at timed intervals.

PRKN gene was chosen for KO pig production. *PRKN* specific TALEN pairs were transfected in to pig somatic cells with reporter, 2 to 3 days after transfection *GFP* signal released cells were chosen for SCNT without

specific selection processes. Eight cloned piglets were born and named, PDM1 to 8. In three (PDM3; heterozygote, PDM5; homozygote, PDM6; homozygote) out of eight, *PRKN* mutation was confirmed. However, these three KO piglets were dead (PDM3; unknown reason, PDM5; euthanasia because of cleft, PDM6; stillbirth). Nevertheless cell lines from those KO pigs except PDM6 were established and will be employed for re-cloned KO piglets.

In conclusion, I demonstrated that immortalized porcine fibroblasts were successfully established using the human *hTERT* gene and the TALEN pairs enabled gene disruptions in these immortalized cells *in vitro*. Additionally, these transfected cells also develop into the blastocyst stages *via* SCNT. Based on these *in vitro* studies, *SNCA* overexpressed pigs and *PRKN* KO pigs were successfully produced. Furthermore protein analysis, behavioral changes, and brain imaging analysis were scheduled in both *SNCA* overexpressed pigs and *PRKN* KO pigs those were planned to reproduce.

Key words: Transgenic animal, SCNT, gene overexpression, gene KO

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LIST OF ABBREVIATIONS

AC	**Alternating current**
ANOVA	**Analysis of variance**
β-ME	**β-Mercaptoethanol**
bp	**Base pair**
BR	**Blastocyst formation rate**
BSA	**Bovine serum albumin**
Cas9	**CRISPR associated protein 9**
cDNA	**Complementary DNA**
CMAH	**Cytidine monophosphate-N-acetylneuraminic acid hydroxylase like protein**
CMV	**Cytomegalovirus**
COC	**Cumulus oocyte complex**
CR	**Cleavage rate**
CRISPR	**Clustered regularly interspaced short palindromic repeats**
DC	**Direct current**
DMEM	**Dulbecco's modified eagle's medium**
DNA	**Deoxyribonucleic acid**
DNMT	**DNA methyltransferase**
dNTP	**Deoxynucleotide**

DSB	**Double strand break**
eBFP	**Enhanced blue fluorescence protein**
eGFP	**Enhanced green fluorescence protein**
ELISA	**Enzyme linked immunosorbent assay**
eRFP	**Enhanced red fluorescence protein**
ESC	**Embryonic stem cell**
ET	**Embryo transfer**
FACS	**Fluorescence activated cell sorting**
FBS	**Fetal bovine serum**
fPCR	**Fluorescence PCR**
FSH	**Follicular stimulating hormone**
g	**Acceleration of gravity**
GAPDH	**Glyceraldehyde 3-phosphate dehydrogenase**
GFP	**Green fluorescence protein**
GGTA1	**α1,3-Galactosyltransferase**
GLUT	**Glucose transporter**
HEPES	**N-[2-hydroxyethyl]piperazine-n'-[2-ethanesulfonic acid]**
h	**Hour**
HR	**Homologous recombination**
hTERT	**Human telomerase reverse transcriptase**
huKO	**humanized Kusabira Orange**
IRES	**Internal ribosome entry site**

ITS	**Insulin transferrin selenium**
IVC	***In vitro* culture**
IVM	***In vitro* maturation**
KO	**knockout**
kV	**Kilo voltage**
LDHA	**Lactate dehydrogenase A**
LH	**Luteinizing hormone**
MACS	**Magnetic activated cell sorting**
MW	**Molecular weight**
NEAA	**Non-essential amino acid**
Neu5Gc	**N-glycolylneuraminic acid**
NHEJ	**Non-homologous end joining**
PA	**Parthenogenetic activation**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
PD	**Parkinson's disease**
PFF	**Porcine follicular fluid**
PRKN	**Parkin**
P/S	**Penicillin/streptomycin**
PVA	**Polyvinyl alcohol**
PVP	**Polyvinyl pyrrolidone**
PZM	**Porcine zygote medium**

RFP	**Red fluorescence protein**
RGEN	**RNA guided endonuclease**
RMCE	**Recombinase mediated cassette exchange**
RNA	**Ribonucleic acid**
RTA	**Relative telomerase activities**
RT-PCR	**Reverse transcriptase-polymerase chain reaction**
SCNT	**Somatic cell nuclear transfer**
SNCA	**α-Synuclein**
T7E1	**T7 endonuclease 1**
TALEN	**Transcription activator-like effector nuclease**
TALP	**Tyrode's albumin lactate pyruvate**
TRAP	**Telomeric repeat amplification protocol**
UPDRS	**Unified Parkinson's disease rating scale**
UV	**Ultraviolet**
ZFN	**Zinc finger nuclease**

PUBLICATION LISTS

PUBLICATION PAPERS

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Hypothesis and Purpose

In this thesis, I hypothesized that several well-developed transgenic techniques in mice (e.g., overexpression by random insertion or KO by TALEN transfection) could also be applicable to establish porcine transgenic cell lines. Moreover, feasibility of transgenic piglet production *via* the same techniques was proved. For this hypothesis, three aims as below were investigated in my thesis.

First, in Chapter I of PART III., to investigate overexpression and KO in porcine cells. *hTERT* genes were randomly inserted in cultured porcine cells. After confirmation of immortalization characteristics of transfected cell lines, two candidate genes, *CMAH and GGTA1*, were to choose for KO sites. Embryonic development of those immortalized and KO cells were to evaluate for further use of those cells as SCNT donors.

Second, in Chapter I of PART IV., to produce PD model pigs, *SNCA* was to randomly insert into normal porcine cells then, conduct the SCNT with the transfected cells. The potentials of produced piglets as PD model would be evaluated by protein and behavioral analyses.

Lastly, in Chapter II of PART IV., to evaluate the feasibility of KO *via* TALEN for PD model, *PRKN* KO cells with specific TALEN pairs were to establish. SCNT would be applied to generate KO cloned piglets.

PART I

LITERATURE REVIEWS

1. Genome engineering

Engineered nucleases recognize the target locus and induce DSB in genomic DNA (Bitinaite *et al.*, 1998; Christian *et al.*, 2010; Marraffini and Sontheimer, 2010). DSB is one of a disaster for cell, without fast recovery damaged cell goes to apoptosis. DSB repair machineries in cells recover the integrity of the genome by two major repair pathway, those names are NHEJ and HR in eukaryotic cells.

NHEJ is a fast, simple, efficient repair pathway with error prone. DNAs were repaired without any complementary sequences in this repair process. Several fragments of DNA were inserted or deleted by chance. Normally, this causes frame shifted from damaged sites, and then truncated none functional protein is translated. This is called loss of function mutation.

HR is error free repair machinery, because damaged DNA is copied from homologous fragment of DNA, which is artificially inserted or naturally existed (e.g., sister chromatid). Homologous sequences flanked expected genes are exactly introduced to the damaged site of genome, which causes not only KO target genes but also overexpress introduced genes coincidentally.

2.1. ZFN

ZFN is artificially constructed restriction enzyme, made by conjugation of DNA binding domain of zinc finger protein and Fok1 endonucleases (Kim *et al.*, 1996). The DNA binding domain of zinc finger can be modified to target expected DNA sequences. Thus zinc fingers detect desired site of genomic DNA and Fok1 endonucleases induce DSB in the target site (Figure 1A).

2.2. TALEN

TALEN is made by fusion of TAL effector DNA binding protein and Fok1 endonuclease. TAL effector DNA binding sites are modified easily to bind exact site of genome. Then Fok1 endonuclease can induce DSB in any desired location of genomic DNA (Figure 1B) (Christian *et al.*, 2010).

2.3. RGEN

This novel gene editing tools were developed from bacteria adaptive immune machinery. The Cas9 protein forms a sequence specific endonuclease with two RNAs (one for guide target site; named as crRNA, another for combine crRNA to Cas9 protein, named as tracrRNA) (Pennisi, 2013). Unlike other genome engineering tools (ZFN and TALEN), RGEN has minimal restriction. This RGEN normally recognize 23 bps in length and ends with two guanines (GG). However theoretically, GG sequences

appear every 8 bps in genome that makes this no more limitation for selection target sequences. Moreover, non-ambiguous interactions between crRNA and target DNA sequences, which is expected to provide high specificity (Figure 1C).

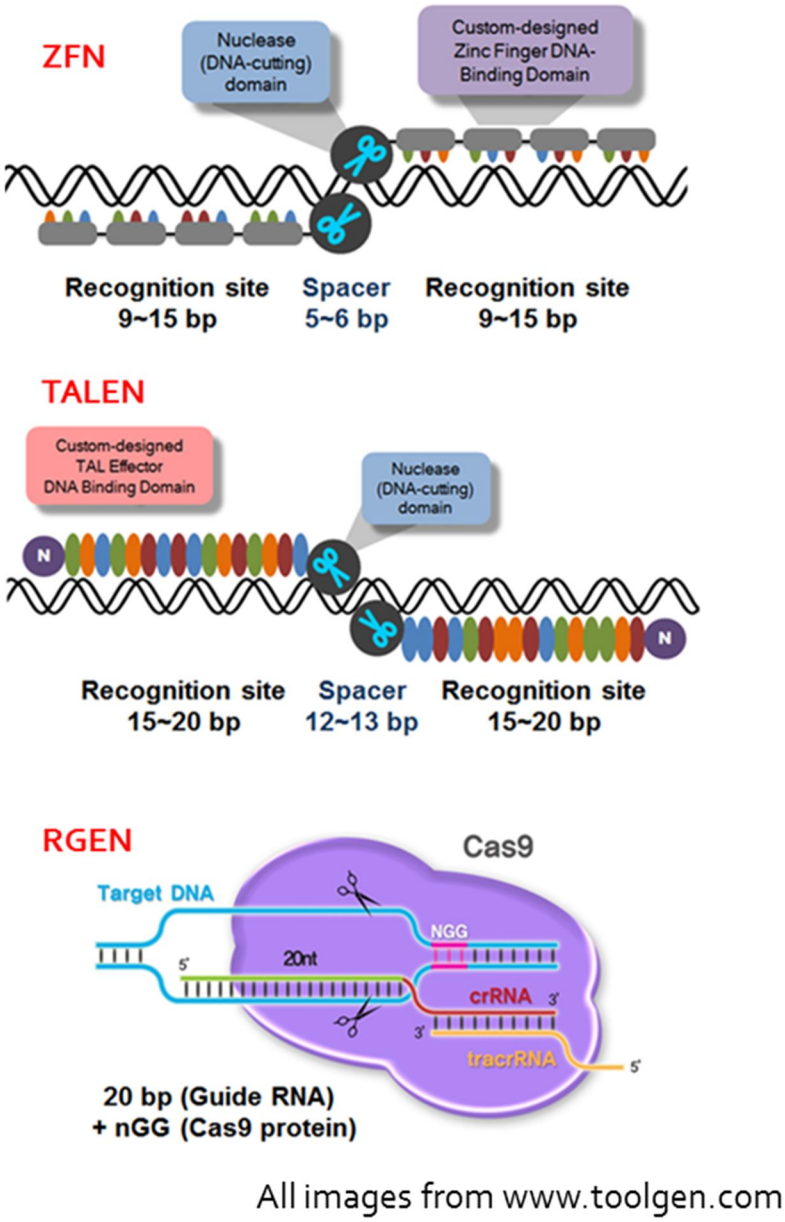


Figure 1. Representative images for genome engineering. (A) Zinc finger nuclease (ZFN). (B) Transcription activator-like effector nuclease (TALEN). (C) RNA guided endonuclease (RGEN).

2. SCNT

Briggs and King cloned tadpoles (Briggs and King, 1952). They used blastomeres from 8-16 thousands embryos as a donor cells for SCNT. They produced 27 tadpoles from 104 SCNT trials, but those few surviving tadpoles were abnormal. So they thought that cloned animals from fully differentiated cells are impossible. However lots of scientist noticed the possibilities of animal cloning. Ten years later, Gurdon finally produced cloned frogs (Gurdon, 1962).

The first mammal Dolly, the sheep, was cloned from fully differentiated adult mammary cell in 1996 by Wilmut (Campbell *et al.*, 1996), lots of mammals including, mouse (Wakayama *et al.*, 1998), cattle (Cibelli *et al.*, 1998), goat (Baguisi *et al.*, 1999), pig (Polejaeva *et al.*, 2000), gaur (Lanza *et al.*, 2000), mouflon (Loi *et al.*, 2001), cat (Shin *et al.*, 2002), rabbit (Chesne *et al.*, 2002), mule (Woods *et al.*, 2003), rat (Zhou *et al.*, 2003), horse (Galli *et al.*, 2003), dog (Lee *et al.*, 2005a), ferret (Li *et al.*, 2006), and wolf (Kim *et al.*, 2007) are produced by SCNT (Table 1).

SCNT is well developed laboratory technique for creating cloned embryos, which can be applied in therapeutic, pharmaceutic, conservative and transgenic cloning. Briefly introducing SCNT, fully differentiated cells were injected into the perivitelline spaces of enucleated matured oocytes. Followed by the nucleus of somatic cells are reprogrammed by the

cytoplasm of oocytes. Reconstructed embryos then transferred into the uterus of recipients.

In therapeutic cloning, patient derived somatic cells are introduced into the oocytes. SCNT derived embryos are dissected and cultured under appropriate culture conditions, and then ESCs or cells derived from embryos can be used for therapeutic purpose. SCNT derived embryos can provide cells which are almost same characteristics of patients.

In conservative cloning, SCNT can be used for rescuing some endangered or already extinct animals. In previous studies, endangered wolves were cloned by using SCNT (Kim *et al.*, 2007). Also cells from post mortem wolves could be used as donor cells for SCNT (Oh *et al.*, 2008). Furthermore frozen cells were used for SCNT, and healthy mice were produced (Wakayama *et al.*, 2008). Resurrection of extinct animals (e.g., mammoths) might be possible in close future.

In pharmaceutical cloning, lots of valuable substances, such as; human factor VIII (Paleyanda *et al.*, 1997), hemoglobin (Swanson *et al.*, 1992), and von Willebrand factor (Lee *et al.*, 2009) could be obtained from transgenic animals *via* SCNT.

Lastly, in transgenic cloning, SCNT has lots of advantages. First of all, SCNT reduce time and costs for making transgenic animals. When using strictly confirmed transgenic donor cells, all pups are transgenic animals. At

least one breeding process is needed if using another method (e.g., blastocyst microinjection) for producing transgenic animals.

Especially in livestock, SCNT is the best choice technique to produce cloned livestock because of no existence of ESCs to date.

Table 1. Lists of animals that have been firstly cloned

Species	Years	Donor cell types	References
Sheep	1996	Adult mammary epithelial	(Wilmut *et al.*, 1997)
Cattle	1998	Fetal fibroblast	(Cibelli *et al.*, 1998)
	1998	Adult oviduct epithelial	(Kato *et al.*, 1998)
Mouse	1998	Adult cumulus	(Wakayama *et al.*, 1998)
	1999	Embryonic stem cell	(Wakayama *et al.*, 1999)
Goat	1999	Fetal fibroblast	(Baguisi *et al.*, 1999)
Pig	2000	Adult cumulus	(Polejaeva *et al.*, 2000)
Gaur	2000	Adult fibroblast	(Lanza *et al.*, 2000)
Mouflon	2001	Adult granulosa	(Loi *et al.*, 2001)
Cat	2002	Adult cumulus	(Shin *et al.*, 2002)
Rabbit	2002	Adult cumulus	(Chesne *et al.*, 2002)
Rat	2003	Fetal fibroblast	(Zhou *et al.*, 2003)
Mule	2003	Fetal fibroblast	(Woods *et al.*, 2003)
Horse	2003	Adult fibroblast	(Galli *et al.*, 2003)
Dog	2005	Adult fibroblast	(Lee *et al.*, 2005a)
Ferret	2006	Adult cumulus	(Li *et al.*, 2006)
Wolf	2007	Adult fibroblast	(Kim *et al.*, 2007)

3. Transgenic cloned pigs

Pigs are not only considered important livestock but also necessary animal models in biomedical research (Matsunari and Nagashima, 2009). Velocity of transgenic mice production and gene modification technologies is untraceable. However differences between humans and mice prevent from utilizing the data those were obtained from mice experiments into therapeutic realm directly. In contrast, pigs have many anatomical and physiological similarities to those of humans. Their physiological sizes of organs are also similar with human organs (Matsunari and Nagashima, 2009). Additionally pigs have another good reasons for research area; 1. Less ethical problems were emerged when it compared to other animals because of industrial animals. 2. Multipara characteristic is fit for large reproducibility in industrial areas. 3. Base facilities are already well established. Of those reasons, pigs are promising animals in transgenic animal production research areas.

After the first cloned pig was reported (Polejaeva *et al.*, 2000), considerable research have been accomplished to produce disease model transgenic, reporter gene transfected, and xeno-transplantation model transgenic pigs.

First, in case of disease model transgenic pigs, porcine retinitis pigmentosa disease models were established (Petters *et al.*, 1997), Huntington disease

model (Uchida *et al.*, 2001), cardiovascular disease model (Lai *et al.*, 2006), cystic fibrosis model (Rogers *et al.*, 2008b; Rogers *et al.*, 2008a), and Alzheimer's disease animal model (Kragh *et al.*, 2009)were also produced. Other disease model was diabetes mellitus model which was characterized by high blood glucose levels and complications, such as; diabetic retinopathy, nephropathy and neuropathy (Wild *et al.*, 2004). Transgenic cloned pigs carrying a mutant *human hepatocyte nuclear factor* (*HNF*)-1α were created (Umeyama *et al.*, 2009).

Second, reporter gene inserted transgenic pigs were also largely reported. These pigs are highly useful in research that requires tracking of transplanted cells or tissues (Matsunari and Nagashima, 2009). *eGFP* inserted transgenic pigs were produced by many researchers (Park *et al.*, 2001; Kurome *et al.*, 2006; Lai *et al.*, 2002b; Hofmann *et al.*, 2003; Hyun *et al.*, 2003; Whitelaw *et al.*, 2004; Lee *et al.*, 2005b; Naruse *et al.*, 2005; Webster *et al.*, 2005; Yong *et al.*, 2006; Brunetti *et al.*, 2008). *eGFP*, *eRFP*, and *eBFP* inserted transgenic pigs were also established in 2005 (Webster *et al.*, 2005). Finally, due to cytotoxicity of the *RFP*, *huKO* gene was newly developed from coral stone *Fungia concinna* (Matsunari *et al.*, 2008).

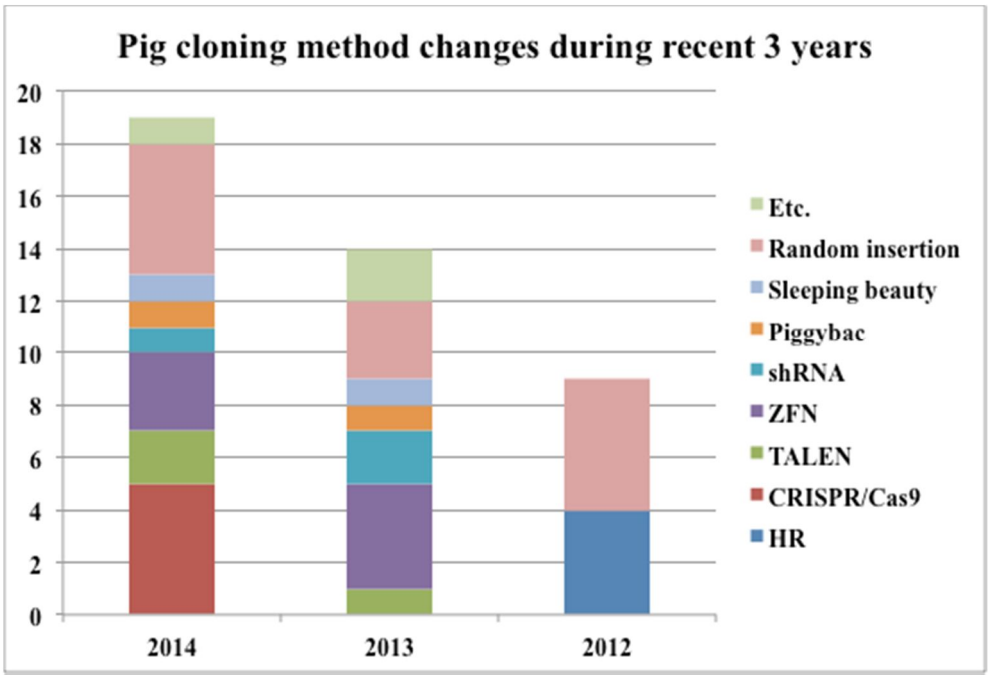
Third, bioreactor pigs were also developed for pharmaceutical reasons. Human factor VIII producing pigs (Paleyanda et al., 1997), hemoglobin producing pigs (Swanson et al., 1992), and von Willebrand factor secreting pigs were established by Lee *et al.*, in 2009.

Finally, pigs can be used as xenotransplantation models due to shortage of organs for human patients. In order to accomplish successful organ graft pigs to humans, organ rejection should be solved. To overcome rejection, multiple gene modification is necessary. Representatively, *GGTA1* gene KO pigs were produced (Lai *et al.*, 2002a). This gene was thought that major factor of hyperacute rejection. Based on this, other genes were rapidly modified in transgenic pig research (Takahagi *et al.*, 2005; Le Bas-Bernardet *et al.*, 2011; Mohiuddin *et al.*, 2011). Production of multi gene modified pigs is time consuming, due to longevity to reach puberty and of reproductive cycle. SCNT could solve these limitations. Obtaining transgenic cells from fetuses or neonates, then repeated transfection to those harvested cells could be possible. Using those transfected cells for donors to SCNT could improve the speed of multi gene modified pig generations. Also, developed gene handling techniques such as; 1. Polycystronic gene regulation by 2A system, 2. RMCE based exact target gene modulation, 3. Transposable elements to improving genes insertion into genomic DNAs, 4. Small hairpin RNAs to knock down target genes, and 5. Gene editing tools (ZFN, TALEN, or RGEN) to KO target genes are combined very fast during recent years (Table 2).

In table 2, newly emerging technologies were introduced in pig production area with speed. Proportions of gene editing tools to produce cloned pigs are greatly increased from 2013. Interestingly, among much

changes in methods to use pig production criteria, transposable elements were constantly used in recent 3 years.

Table 2. Pig cloning methods changes during recent 3 years



PART II

GENERAL METHODOLOGY

Chapter I. Experimental protocols for porcine cloning

1. Chemicals and materials

All chemicals were obtained from Sigma-Aldrich Co. LLC. (Missouri, USA) unless otherwise stated.

2. Oocyte preparation

Pig ovaries were collected from a local abattoir (Wooseok food Inc., Kyeonggido, Korea) and transported to the laboratory within 3 h in physiological saline. Follicular fluid was aspirated from follicles 3 to 6 mm in diameter using an 18-gauge needle attached to a 10 ml syringe (Hwajin Ltd., Chungnam, Korea), transferred to 50 ml conical tubes (Catalog number; 50050, SPL Lifesciences, Kyeonggido, Korea), and incubated at 39°C. After 15 min of sedimentation, the supernatant was discarded. The sediments were transferred to a 100 mm Petri dish (Catalog number; 10090, SPL Lifesciences) and washed with physiological saline containing 1% (v/v) P/S or porcine washing media (Table 3). COCs with intact, unexpanded cumulus cell layers and homogeneous cytoplasm were selected for further culturing. The COCs were cultured with IVM medium (Table 4) at 39°C under 5% CO_2 in air for 22 h with 0.5 μg/ml of FSH and 0.5 μg/ml of LH. Next, the COCs were washed with Medium 199, transferred to hormone free IVM medium, and cultured at 39°C under 5% CO_2 and 5% O_2 in air for

another 22 h for final oocyte maturation. Cumulus cells were removed from the oocytes by repeated pipetting in Tyrode lactose (TL) medium supplemented with 10 mM HEPES and 0.3% (w/v) PVP (HEPES-TL-PVP) medium with 1 mg/ml hyaluronidase (This is the full name of TALP.).

3. Donor cell preparation

Pig fibroblast or kidney cells, which were used as control cells, were isolated and cultured. Tissues were washed 3 times in PBS and then chopped into small pieces in a 60 mm dish with trypsin. Trypsinized tissues were then incubated for 30 min at 37^{o}C. Well dissociated tissues were centrifuged at 1,500 rpm for 2 min. The supernatant was discarded and the pellet was resuspended with PBS then centrifuged at 1,500 rpm for 2 min. These procedures were repeated 2 times. Finally, the supernatant was discarded and the pellet was resuspended in DMEM (Gibco, California, USA) supplemented with 20% FBS (Gibco), 1% P/S (Gibco), 1% NEAA (Gibco) and 100 mM β-ME by inverting the tube several times. The cells resuspended in this medium were held at room temperature (around 25^{o}C) for 5 min, and then the suspension was transferred into a cell culture dish for approximately 10 days with culture medium changes every 2-3 days. These primary cells were cultured, expanded and frozen at -196^{o}C for further use. The cell cultures were maintained in DMEM with 20% FBS, 1% P/S, 1% NEAA and 100 mM β-ME.

4. SCNT

After 40 h of *in vitro* matured cumulus oocyte complexes (COCs) were move to 0.1% (v/v) hyaluronidase in TALP (Table 5) media. The cumulus cells were removed from oocytes by pipetting. Only denuded oocytes with homozygous cytoplasm were stained with 5 μg/ml bisbenzimide (Hoechst 33342) for 10 min. Before starting enucleation process, well stained oocytes were moved to TALP for washing.

For enucleation, the stained oocytes were transferred to 5 mg/ml cytochalasin B containing TALP media for enucleation. Observe the oocytes under the inverted microscope equipped with epifluorescence. Oocyte was held with a holding micropipette and the zona pellucida (ZP) was punctuated with an aspiration pipette to approach the polar body and nucleus of cytoplasm with a micromanipulator (Nikon-Narishige, Tokyo, Japan). The first polar body and adjacent cytoplasm containing the metaphase II chromosomes were enucleated by aspiration pipette.

During injection step, prepared donor cells were suspended in TALP, and enucleated oocytes were also transferred to another TALP media without cytochalasin B. By using a same aspiration pipette, a cell with smooth cellular membrane was injected into the perivitelline space of an enucleated oocyte. The pore that was made in enucleation step was preferred to injection site.

At the fusion step, the couplets were serially equilibrated with fusion solution (Table 7) for several min and fused in 20 μl fusion solution droplet by applying an AC field of 5 V for 2 sec, followed by a DC pulses of 35 V for 2 sec, and frequency of 1 MHz for 30 μsec one pulse with 0.1 sec intervals using an electrical pulsing machine (LF101 Electro Cell Fusion Generator; NepaGene, Chiba, Japan).

Activation step was followed within 30 to 60 min after fusion, fused oocytes were serially equilibrated with activation solution (Table 8) for several min and transferred to a chamber containing two electrodes overlaid with activation solution and activated with a single DC pulse of 1.5 kV/cm for 60 μsec using a BTX Electro-Cell Manipulator 2001 (BTX Inc., California, USA).

Last, post-activation step, fully activated embryos were immediately transferred into PZM-3 (Table 6) or PZM-5 supplemented with 5 μg/ml cytochalasin B for 4 h for post-activation. The activated embryos were washed three times with PZM and transferred into the fresh PZM media under mineral oil, and then cultured at 39°C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2 for up to 7 days.

Table 3. Components of porcine oocyte washing media

Component	Catalog No.	M.W.****	mM
Medium 199 powder*			1 pack/l
NaOH	S8045	40.00	2 tablets/l
$NaHCO_3$	S5761	84.01	2
HEPES	H6147	238.31	10
PVA**	P8136		0.30%
P/S***			1%

*Medium 199 powder (Invitrogen; 31100-035, California, USA)
**Polyvinyl alcohol
***Penicillin streptomycin (Invitrogen; 15070-063, California, USA)
****Molecular weight

Table 4. Components of porcine IVM media

Component	Catalog No.	M.W.*****	mM
Medium 199 liquid*			500 ml
$NaHCO_3$	S5761	84.01	26.2
Glucose	G7021	180.16	3.05
Na pyruvate	P4562	110.04	0.91
Ca lactate	L4388	103.1	2.92
L-cystein	C7477	157.62	0.57
Kanamycin	K1377	582.58	0.075
PFF**			10% (v/v)
FSH***			0.5 μg/ml
LH****			0.5 μg/ml

*Medium 199 liquid (Invitrogen; 11150-059, California, USA)
**Porcine follicular fluid
***Follicular stimulating hormone
****Luteinizing hormone
*****Molecular weight

Table 5. Components of TALP

Component	Catalog No.	M.W.***	mM
NaCl	S5886	58.44	114
KCl	P5405	74.55	3.1
$CaCl_2 \cdot 2H_20$	C7902	147.02	2.1
$MgCl_2 \cdot 6H_20$	M2393	203.31	0.4
$NaH_2PO_4 \cdot H_20$	S3522	138	0.3
$NaHCO_3$	S5761	84.01	2
Na pyruvate	P4562	110.04	0.2
Glucose	G7021	180.16	5
HEPES	H6147	238.31	10
Kanamycin	K1377	582.58	0.17
PVP*	P0930	40.00	0.30%
BSA**	A6003		3 mg/ml
Na lactate	L7900	112.06	10

*Polyvinyl pyrrolidone
**Bovine serum albumin (Fatty acid free, fraction V)
***Molecular weight

Table 6. Components of PZM-3

Component	Catalog No.	M.W.****	mM
NaCl	S5886	58.44	108.00
KCl	P5405	74.55	10.00
KH_2PO_4	*P5379*	136.09	0.35
$MgSO_4 \cdot 7H_20$	M7774	246.5	0.40
$NaHCO_3$	S5761	84.01	25.07
Na-Pyruvate	P4562	110.04	0.20
Ca-Lactate	L4388	103.1	2.00
L-glutamine	G8540	146.15	1.00
Hypotaurine	H1384	109.1	5.00
BME amino acid*	B6766		20 ml/l
MEM NEAA**	M7145		10 ml/l
Kanamycin	K1377	582.58	75 mg/l
BSA***	A6003		3 mg/ml

*Basal medium eagle amino acids
**Minimum essential medium non-essential amino acid
***Bovine serum albumin (Fatty acid free, fraction V)
****Molecular weight

Table 7. Components of embryo fusion media

Component	Catalog No.	M.W.**	mM
Mannitol	M1902	182.17	280
$MgSO_4$	M2643	120.4	0.1
PVA*	P8136	238.31	0.01% (w/v)
HEPES	H6147	238.3	0.5
$CaCl_2$	C4901	111	0.001

*Polyvinyl alcohol
**Molecular weight

Table 8. Components of embryo activation media

Component	Catalog No.	M.W.**	mM
Mannitol	M1902	182.17	280
$MgSO_4$	M2643	120.4	0.15
PVA*	P8136	238.31	0.01% (w/v)
HEPES	H6147	238.3	0.5
$CaCl_2$	C4901	111	0.05

*Polyvinyl alcohol
**Molecular weight

5. ET and pregnancy test

The surrogate mother was restrained and preanesthesia was induced by injection of ketamine (10 mg/kg; Yuhan, Seoul, Korea) and xylazine (1 mg/kg; Bayer, Leverkusen, Germany) into an ear vein as previously described (Trim and Gilroy, 1985). The anesthetized pig was placed on a surgery table in a ventrodorsal posture. General anesthesia was maintained with isoflurane by concentration of 2.0 MAC (Hana Pharm, Seoul, Korea) under the supervision of a veterinarian.

The recipient was ventrodorsally laid on surgical table, and uterus and ovaries were taken out from abdomen through midline incision.

Reconstructed embryos were loaded into a Tomcat catheter (Catalog number; 8890703021, Covidien, Dublin, Ireland) with TALP medium equilibrated in 5% CO_2 with air. Only 2- or 4-cell stage embryos were transferred into surrogate mothers those were 2 to 3 days after ovulation. The embryos were placed into the oviducts of each surrogate animal through a small puncture made with a suture needle (Catalog number; 6307-71, Covidien). The empty tomcat catheter was removed carefully, and then put uterus and ovaries into the abdominal cavity, followed by suture the opened abdomen.

6. Analysis of cloned animals

6.1. PCR and RT-PCR

For PCR analysis, genomic DNA was extracted with the G-spin Genomic DNA Extraction Kit (iNtRON Biotechnology, Gyeonggido, Korea) according to the manufacturer's protocol. Amplification of target genes was achieved using Maxime PCR PreMix (i-StarTaq; iNtRON).

For RT-PCR, total RNAs were extracted to analyze gene expression in the immortalized cells by using the easy-spin Total RNA Extraction Kit (iNtRON). Then, cDNAs were synthesized using Maxime RT Premix (iNtRON) according to the manufacturer's protocol.

6.2. Motor scoring analysis

The scoring analysis table for pigs was based on UPDRS (Mitchell *et al.*, 2000). This newly constructed scoring analysis table has 21 categories each with a score from 0 to 3, representing none to severe, respectively (Table 9). Thus, the sum total of scores ranged from 0 to 63 (Moon *et al.*, 2014b).

Table 9. Proposed motor scoring system for a porcine PD model based on the UPDRS in human PD patients

No.	Categories	Score 0 (Normal)	Score 1 (Mild)	Score 2 (Moderate)	Score 3 (Severe)
Tremor					
1	Tremor at rest	None	Mild	Moderate	Severe
2	Tremor in moving	None	Mild	Moderate	Severe
Loss of automatic movements					
3	Drooling	Never	Sometimes	Frequent	Always
4	Amount of food intake	Normal	Decreased to 70% of normal	Decreased to 40~70% of normal	Decreased to below 40% of normal
5	Amount of water intake	Normal	Decreased to 70% of normal	Decreased to 40~70% of normal	Decreased to below 40% of normal
6	Number or speed of nictation	Normal	Decreased to 70% of normal	Decreased to 40~70% of normal	Decreased to below 40% of normal
7	Movement	Normal	Decreased to 70% of normal	Decreased to 40~70% of normal	Decreased to below 40% of normal
8	Response to pungent odor	Moves immediately	Sometimes	Rarely	No response
Slowed motion (bradykinesia)					
9	Contact response	React	Sometimes	Rarely	No reaction
10	Speed of walking	Normal	Decreased to 70% of normal	Decreased to 40~70% of normal	Decreased to below 40% of normal
Impaired posture and balance					
11	Balance of body in walking	Normal	Decreased to 70% of normal	Decreased to 40~70% of normal	Decreased to below 40% of normal
12	Reaction velocity for threat	Normal	Decreased to 70% of normal	Decreased to 40~70% of normal	Decreased to below 40% of normal
13	Balance of body in normal status	Normal	Decreased to 70% of normal	Decreased to 40~70% of normal	Decreased to below 40% of normal
Impaired response to external stimuli					
14	Curiosity for new object	Curious and play	Sometimes	Rarely	No reaction
15	Escaping	None	1 time per week	2~6 times per week	Everyday
16	Change in grunting sound	Normal	Decreased to 70% of normal	Decreased to 40~70% of normal	Decreased to below 40% of normal
17	Response to spotlight	Moves immediately	Sometimes	Rarely	No response
18	Response to loud sound	React	Sometimes	Rarely	No response
19	Crying sound before eating	Normal	Decreased to 70% of normal	Decreased to 40~70% of normal	Decreased to below 40% of normal
20	Foot retrieval from holding devices	Normal	Decreased to 70% of normal	Decreased to 40~70% of normal	Decreased to below 40% of normal
21	Response to attachable materials	React	Sometimes	Rarely	No response

PART III

APPLICATION OF VARIOUS GENE MODULATING TECHNOLOGIES IN *IN VITRO*

Chapter I. Production of *CMAH* KO preimplantation embryos derived from immortalized porcine cells *via* TALEN.

1. Introduction

Pigs are considered to be good biomedical models for topics such as xenotransplantation because of their many physiological similarities with humans (Matsunari and Nagashima, 2009; Lai *et al.*, 2002a; Aigner *et al.*, 2010; Kwon *et al.*, 2013). SCNT with genetically modified somatic cells has been used to generate pig models *via* transgenesis (Wolf *et al.*, 2001). Typical gene modifications are ectopic expression or KO of target genes (Liu *et al.*, 2013; Lutz *et al.*, 2013). While many cloned piglets have been produced using ectopic expression, only three kinds of KO piglets (alpha-galactosidase, cystic fibrosis and IL2 receptor) using HR have been born (Lai *et al.*, 2002a; Rogers *et al.*, 2008b; Rogers *et al.*, 2008c). Developing KO pig models has been hampered to date because fibroblasts generally have a limited life span during IVC and because of the low efficiency of the HR process (Kwon *et al.*, 2013). To overcome these two issues, immortalization of fibroblasts and more efficient KO protocols are needed. For immortalization, several genes such as *BMI, SV40LT* and *hTERT* can be transfected into cells. In previous studies, *SV40LT* and *hTERT* were used to immortalize porcine cells (Sagong *et al.*, 2012; Saito *et al.*, 2005; Oh *et al.*, 2007; Meng *et al.*, 2010).

TALEN is an emerging high end technology used to create targeted double stranded breaks in DNA (Kim *et al.*, 2013b). Currently, TALEN has been employed for genome editing, resulting in target gene deletion or insertion in human, mouse and rat cells (Kim *et al.*, 2011; Zhu *et al.*, 2013; Ding *et al.*, 2013; Tong *et al.*, 2012; Panda *et al.*, 2013). Application of TALEN to cells of large animals like pigs could more efficiently generate KO cell lines and thus help to elucidate the underlying molecular processes (Carlson *et al.*, 2012). After establishing KO cell lines, the cell nuclei could potentially be reprogrammed in enucleated oocytes and produce KO cloned offspring.

In this study, to prove TALEN mediated KO, I elected to delete the *CMAH* gene, which is another important cell surface glycoprotein with alpha-galactosidase for xenotransplantation pig models and then gene KO cells were used for feasibility of embryonic development *via* SCNT. Here I hypothesized that using immortalization and TALEN approaches together in porcine cells, these could serve as practical *in vitro* models of genome editing.

2. Materials and methods

2.1. Primary cell culture and maintenance

Male fetal fibroblasts from one miniature pig fetus, which were used as control cells, were isolated and cultured. Euthanized fetus was dissected into 3 parts: head, body and tail. Just the body parts of fetuses were washed 3 times in PBS and then following the protocol previously established in our studies (Moon *et al.*, 2014a).

2.2. Immortalization

For immortalization, *hTERT* (from Addgene, Plasmid #12245) were amplified by PCR. Purified *hTERT* fragments were inserted in p*CMV-IRES-DsRed* vectors, which were purchased from Clontech (Seoul, Korea.). p*CMV-hTERT-IRES-DsRed* plasmids were transfected into male fetal fibroblasts which was same cells as control cells using FugeneHD (Figure 2A). Two days after transfection, 1000 μg/ml, neomycin (G418; Gibco) were treated for 7 days to isolate the transfected cells and then growing cells to neomycin resistance were sub-cultured (Figure 2).

2.3. Cell properties

2.3.1. Doubling time

Controls and immortalized cells were plated in 12-well plates at 4×10^4 cells/well. Every 24 h, cells in four of the wells were trypsinized and cell

numbers were calculated manually under a hemacytometer. Then, the doubling time was calculated using the doubling time online calculator (http://www.doubling-time.com/compute.php) every 3 passages up to passage 24 (Widera *et al.*, 2009).

2.3.2. Cell Size

Images from trypsinized cells on the hemacytometer were taken under a microscope (×200). Sizes of one hundred cells were measured by ImageJ (http://rsbweb.nih.gov/ij/) every 3 passages up to passage 24 (Collins, 2007).

2.3.3. PCR

Genomic DNA was extracted with the G-spin Genomic DNA Extraction Kit (iNtRON) according to the manufacturer's protocol. Amplification of target genes was achieved using Maxime PCR PreMix (i-StarTaq; iNtRON). Primer sets, conditions and expected sizes are annotated in Table 10.

2.3.4. Sequencing

Target DNA samples were delivered to a sequencing company (Macrogen Ltd., Seoul, Korea). Briefly, sequencing reactions were performed in the DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD, California, USA) using the ABI BigDye (R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA), following the protocols supplied by the

manufacturer. Single pass sequencing was performed on each template using a selected primer (primer sequences: AACGTTCCGCAGAGAAAA GA). The fluorescent labeled fragments were purified by the method recommended by Applied Biosystems because it removes unincorporated terminators and dNTPs. The samples were subjected to electrophoresis in an ABI 3730xl DNA Analyzer (Applied Biosystems).

2.3.5. Karyotyping

To perform karyotyping, cultured cells were treated as follows. First, 200 μl of colcemid (Gibco) stock solution was added to the culture. Then, the culture was returned to the incubator (37^{o}C, 5% CO_2) for 4 h. After incubation, cells were collected in 15 ml tubes and then centrifuged at 1000 rpm for 10 min. The medium was carefully aspirated, then 5 ml of hypotonic solution (0.075 M KCl) was added and allowed to stand at 37^{o}C for 10 min. Then, 500 μl of Carnoy's fixative (Methanol: Acetic acid = 3:1) was added and mixed by inverting the tube, followed by centrifugation at 1000 rpm for 10 min. The hypotonic solution was aspirated carefully and 3 ml of Carnoy's fixative was added and well mixed. After more than 20 min, the mixture was centrifuged at 1000 rpm for 10 min. The supernatant fixative solution was carefully aspirated till leaving about two times of volume to pellets. The pellet was spread on a prepared glass slide which was then baked at 60^{o}C for 30 min. The slide was treated with 50% H_2O_2 for 3

min, and then baked again at 60°C for 30 min. Finally the slide was stained with the Giemsa stain-GTG banding method. Chromosome imaging were accomplished with the ChIPS-Karyo (Chromosome Image Processing System) (GenDix, Inc., Seoul, Korea).

2.4. Single cell colony formation

Trypsinized cells were placed on the lid of Falcon dish (Catalog number #351006; Falcon, New Jersey, USA) in drops of 20 μl of DMEM containing 15% FBS, 1% P/S, 1% NEAA and 100 mM β-ME. To evaluate single cell colony forming competence, one cell was picked up in a micropipette attached to a micromanipulator. The cell was transferred into a 4 μl drop of DMEM containing 15% FBS, 1% P/S, 1% NEAA and 100 mM β-ME that was covered with mineral oil. After seven days, growing cell colonies were collected and sequentially sub-cultured into 96-, 24- and 6-well plates. Then, cells from the 6-well plates were moved sequentially to 60mm and 100mm dishes.

2.5. Gene expression

Total RNAs were extracted to analyze gene expression in the immortalized cells by using the easy-spin Total RNA Extraction Kit (iNtRON). Then, cDNAs were synthesized using Maxime RT Premix (iNtRON) according to the manufacturer's protocol. Information on primers is listed in Table 11.

Gene expression for *p53, p16, Bax, Bcl-xl, DNMT*1, *DNMT*3a, *DNMT*3b, *GLUT1*, and *LDHA* was measured with a real-time PCR machine (7300 Real-Time PCR System; Applied Biosystems).

2.6. Telomerase activity test

Quantification and characterization of telomerase activity was measured by the TRAP. For this test, TeloTAGGG Telomerase PCR ELISA^{PLUS} (Roche, Basel, Switzerland) kit was used with manufacturer's indications (Mariani *et al.*, 2003). RTA within different samples in an experiment were obtained using the following formula;

RTA = [(AS-AS0)/AS,IS]/[(ATS8-ATS8,0)/ATS3,IS]×100

(AS; absorbance of sample, AS,0; absorbance of heat treated sample, AS,IS; absorbance of internal standard (IS) of the sample, ATS8; absorbance of control template, ATS8,0; absorbance of lysis buffer, ATS8,IS; absorbance of IS of the control template).

2.7. SCNT

Donor cells were subjected to SCNT, which was done following the protocol previously established in our studies (Koo *et al.*, 2009). Briefly, immature oocytes were obtained from pig ovaries and cultured for 40 h to support maturation. The *in vitro* matured (IVM) oocytes were enucleated using an aspiration pipette, then microinjected with a control or transfected

donor cell, fused by electrical stimulation and activated using an electrical protocol. The resulting activated embryos were cultured for seven days. Cleavage and blastocyst stages were observed on Day 2 and 7 of culture, respectively.

2.8. *CMAH* KO using TALE nucleases and magnetic separation

$1x10^6$ immortalized cells were transfected using 30 μl of Turbofect^{TM} (Fermentas Inc., Maryland, U.S.A.) and 10 μg of plasmid DNA at a weight ratio of 45:45:10 (plasmid encoding a TALEN: plasmid encoding the other TALEN: magnetic reporter) according to the manufacturer's protocol (Kim *et al.*, 2011; Kim *et al.*, 2013a). The transfected cells were cultured for two days at 37^{o}C and subjected to magnetic separation. Trypsinized cell suspensions were mixed with magnetic bead conjugated antibody against H-2K^{k} (MACSelect K^{k} microbeads; Miltenyi Biotech, Cologne, Germany) and incubated for 15 min at 4^{o}C. Labeled cells were separated using a column (MACS LS column; Miltenyi Biotech) according to the manufacturer's protocol.

2.9. T7E1 assay

Genomic DNA was extracted using the G-DEX IIc Genomic DNA Extraction Kit (iNtRON) after 3 days of transfection. TALEN target sites were PCR amplified using primer pairs listed in Table 10. The T7E1

analysis was done as described previously (Kim *et al.*, 2009). The amplicons were denatured by heating and annealed to form heteroduplex DNA, which was treated with 5 units of T7E1 (New England Biolabs, Massachusetts, USA) for 20 min at 37^{o}C and then analyzed by 2.5% agarose gel electrophoresis.

2.10. Fluorescent PCR

Carboxyfluorescein (FAM) was labeled on 5' of the forward primer by an oligo synthesis company (Bioneer Corporation, Daejon, South Korea). PCR products were processed for fragment separation by capillary electrophoresis on an ABI 3730xl using POP-7 polymer. The GeneScan Rox500 size standard (Life technologies, California, USA) was run as an internal size marker. Samples were denatured at 95^{o}C for 5 min and run on the Genetic Analyzer. Data were analyzed for allele sizes and peak heights using the pick scanner software v1.0 (Life technologies).

2.11. FACS

CMAH biallelic KO cells were trypsinized and resuspended in staining buffer (0.1% BSA in PBS) to reach a final concentration of 5×10^5 to 1×10^6 cells/ml. The cells were incubated for 20 min on ice with the antibody anti *Neu5G*c (Sialix, Massachusetts, USA). After incubation, the cells were

washed twice with staining buffer and resuspended, and then the stained cells were analyzed by FACS.

2.12. Statistical analysis

All data were analyzed by one way ANOVA followed by Tukey's multiple comparison tests or paired t-test using GraphPad Prism version 5.01 to determine differences among experimental groups. Statistical significance was determined when the p-value was less than 0.05.

3. Results

3.1. Cell properties

3.1.1. Differences between controls and immortalized cells in morphology, doubling times and cell size

After transfection, outgrowing colonized fibroblasts were cultured. Along with immortalization of the cells, their size was reduced (Figure 2D).

Mean doubling time of control and immortalized cells were 46.4±1.1 and 26.9±0.6 h, respectively, and these values were significantly different ($p < 0.05$) (Figure 2B). Mean cell size of the immortalized cells was 17.9±0.2 μm and always less than 20 μm while mean cell size of control cells was progressively increasing until finally these cells entered into replicative senescence. Significant differences in cell size between control and immortalized cells were observed from passage 12 (Figure 2C).

3.1.2. PCR, RT-PCR and Sequencing

Integration and expression of the *hTERT* gene was observed by PCR and RT-PCR in 3 and 18 passages of control and immortalized cells, respectively. PCR and RT-PCR data indicated that the *hTERT* gene was integrated into immortalized cells (Figure 3A and 3B). Also, sequencing results from both PCR amplicons were exactly the same as those inserted sequences from the vector (Figure 8).

3.1.3. Karyotyping

A total of 20 cells in each analysis were subjected on karyotyping. Karyotyping of immortalized cells, prior to passage number 15, revealed normal chromosomes while subsequently abnormal chromosomes were detected in one cells (Figure 2E). Similar observations were made in control cells, indicating that eight cells showed abnormalities (trisomy in chromosome #17) (Figure 2E).

3.2. Single cell colony formation

Immortalized cells could be populated from a single cell in a 100mm dish. This ability was replicated three times more using single cells. However, control cells did not have the ability to be expanded from a single cell in a 100mm dish (Figure 6).

3.3. Gene expression

Gene expression in immortalized cells and control cells are summarized in Figure 3D. In this analysis, tumor suppressor gene (*p53*) expression level was not significantly changed during increasing passage number in immortalized or control cells except passage 6 and 15. Cyclin-dependent kinase inhibitor 2A (*p16*) expression was significantly down regulated during increasing passages (from passage 6) in immortalized cells. Also *Bax*, which is a well-known proapoptotic gene, was significantly down regulated

during increasing passage numbers (from passage 6) in immortalized cells. However, *Bcl-xl*, an antiapoptotic gene, was significantly changed in passage numbers 6 and 18. In analysis of metabolic genes, expression of *GLUT1* and *LDHA* were significantly up regulated in late passage of immortalized and control cells, respectively. Expression of methylation relation genes (*DNMT*1, *DNMT*3a and *DNMT*3b) was not changed (Figure 7).

3.4. Telomerase activity test

RTA of control and immortalized cells were 0.41 ± 0.16 and 5.37 ± 0.09, respectively. Telomerase activity was significantly increased in immortalized cells compared to control cells (Figure 5).

3.5. Preimplantation development of cloned embryos derived from immortalized cells

Development rates were evaluated in three groups: parthenogenetic activated embryos (total numbers of oocytes: 211), SCNT derived embryos using control somatic cells as nuclear donors (total numbers of oocytes: 112) and SCNT derived embryos using immortalized cells as nuclear donors (total numbers of oocytes: 107). Three replicates were done in all three groups. Two days after activation, CRs evaluated under a microscope were 81.6±2.2%, 68.1±0.8% and 71.8±3.6%, respectively. No significant

differences were observed in CR among the three groups. However, significant differences were observed in BRs, which were 32.3±1.2%, 11.5±0.7% and 2.9±0.2%, respectively (Figure 3C).

3.6. *CMAH* KO and SCNT

After transfecting TALEN DNAs, 500 reporter gene positive cells were cultured in a 100 mm dish and grown into colonies; 116 single cell derived colonies were selected. In a T7E1 mutation assay, I found 45 colonies to be mutated (Figure 9). Among these 45 colonies, I subjected to fluorescent PCR for determination of biallelic mutated colonies (Figure 10). Three biallelic mutation colonies with morphologically good cells were finally selected and sequenced for confirmation of biallelic mutation (Figure 4D). In #13, 1 bp insertion and 1bp deletion were found, in # 24, a 282 bps insertion (sequence of 282 bps was noted in Figure 11) and in #26, 2 bps and 8 bps deletions were observed (Figure 4C). In addition, as shown by FACS, *CMAH* expression was removed in all three cell lines (Figure 4E). Furthermore, thirty six cloned embryos derived from *CMAH* KO cells were reprogrammed after insertion into enucleated oocytes, cleaved (91.7%) and developed into a blastocyst (2.8%) (Figure 12).

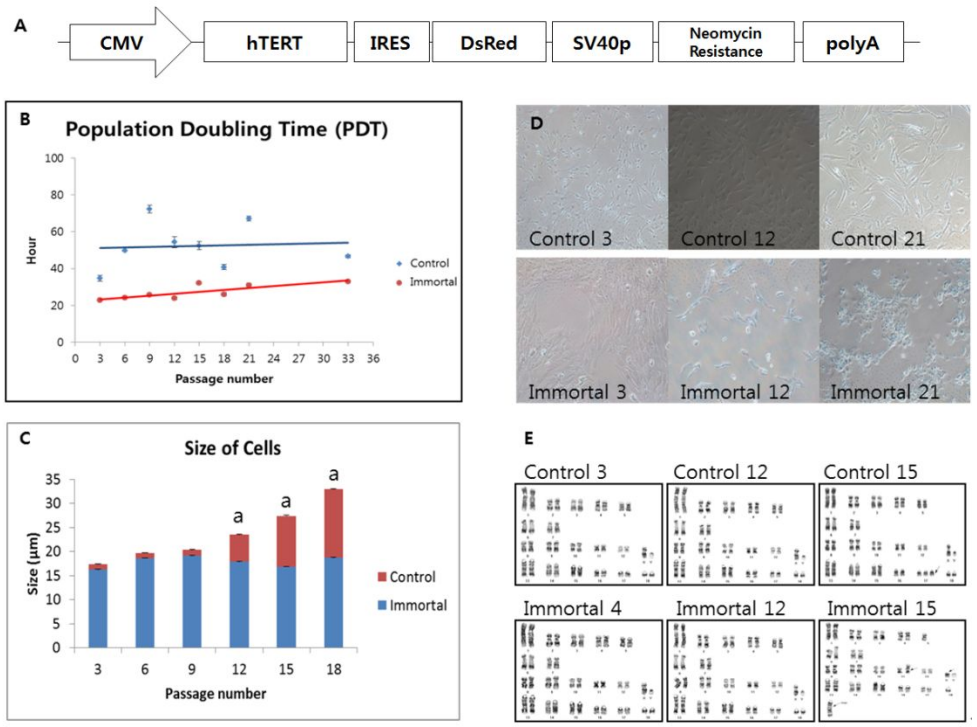


Figure 2. Cellular analysis of porcine immortalized cells. (A) Illustration of p*CMV-hTERT-IRES-DsRed*, (B) Population doubling time, significant differences in doubling time between control and immortalized cell were investigated and those were 46.4±1.1 and 26.9±0.6, respectively. (C) Size differences between control cells and immortalized cells, mean cell size of the immortalized cells were 17.9±0.2 which was constantly under the 20 μm while that of control cells were sequentially increasing in mean cell size and finally these cells were enter into senescence or crisis. Significant differences in cell size between control and immortalized cells were observed from passage number 12. (D) Morphologies of control cells and immortalized cells, numbers represent passages. (E) Results of karyotyping,

both control and immortalized cells showed abnormalities from passage number 15. Arrows indicated the abnormal site in chromosomes.

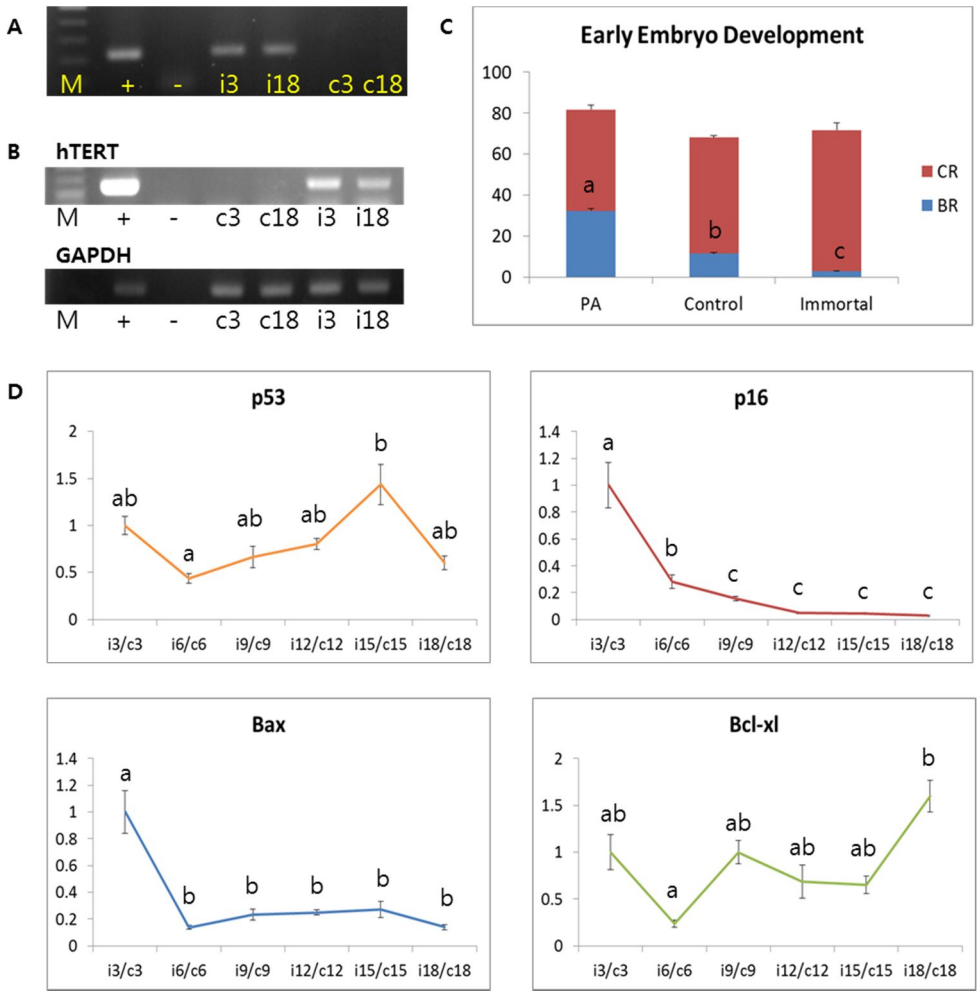


Figure 3. Gene expression in immortalized cells and embryonic development. (A) Detection of *hTERT*, (M; marker, +; positive control vector, -; negative control vector, c; control cells, i; immortalized cells and numbers referred to passages). (B) Expression of *hTERT*, (M; marker, +; positive control vector, -; negative control vector, c; control cells, i; immortalized cells and numbers referred to passages). (C) Early embryonic development, changes among early embryonic development when cells properties were changed into immortal states. Those control and immortal

indicated SCNT results when the donor cell were control and immortalized cells, respectively. CRs were not changed among groups, but BRs were serially significantly decreased among three groups. (D) Gene expression, tumor suppressor gene (*p53*) expression level was not significantly changed during the increasing of passage number in immortalized cells/control cells. Cyclin-dependent kinase inhibitor 2A (*p16*) expression were significantly down regulated during the increasing of passage numbers in immortalized cells/control cells. Also *Bax*, which is well known for proapoptotic gene, were significantly down regulated during the increasing of passage numbers in immortalized cells/control cells. However *Bcl-xl*, antiapoptotic gene, was significantly up regulated during the increasing of passage numbers in immortalized cells/control cells.

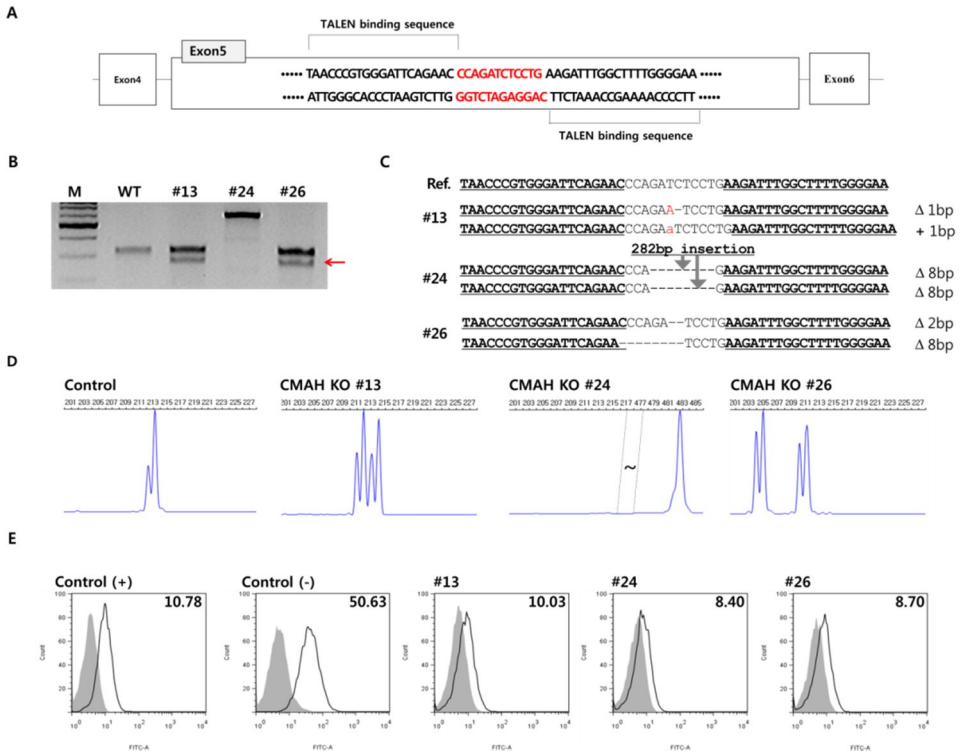


Figure 4. Generating *CMAH* KO cells and its analysis. (A) DNA-binding sequences and the spacer region for *CMAH*-TALEN. (B) T7E1 assays, T7E1 assays were conducted using genomic DNA from three *CMAH* KO clones. The arrow indicates the size (~170 bps) of T7E1 digested DNA fragments. (C) DNA sequences of the *CMAH* locus from each *CHAH* KO clone. '-' denotes deleted nucleotides. Red colored upper case letter and lower case letter sequences represent nucleotide substitutions and insertion, respectively. (D) fPCR assay of the *CMAH* KO clones. (E) FACS analysis of *CMAH* KO clones. The expression level of *Neu5Gc* is detected by anti-*Neu5Gc* antibody on the *CMAH* KO cell membrane. The expression levels of *Neu5Gc* on each *CMAH* KO clone are comparable with control (+).

Control (+): human embryonic kidney cell line; Control (-): non-transfected porcine fibroblasts.

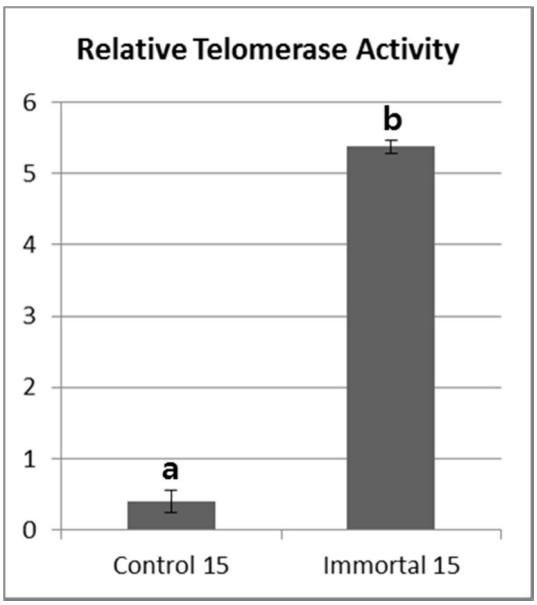


Figure 5. RTA. RTA of control and immortalized cells were 0.41 ± 0.16 and 5.37 ± 0.09, respectively. Telomerase activity was significantly increased in immortalized cells compared to control cells.

Table 10. List of primers

Gene	Primer sequences (5'-3')		Size of PCR product (bp)	GenBank accession no.
	Forward	Reverse		
hTERT	GTGGTGAACTTCCCTGTAGAAGAC	GAAACAGGCTGTGACACTTCAG	250	NC_000005.9
GAPDH	ACCTGCCGTCTGGAGAAACC	GACCATGAGGTCCACCACCCTG	252	AF017079
1st *CMAH*	TTGGTCTTCAGCCCTCATCT	CT*GGTA*GCAAGGGCAGTTTC	743	NM_001113015.1
2nd *CMAH*	TTGGTCTTCAGCCCTCATCT	ATTTAACATTTCCTTACCTGCAC	307	NM_001113015.1
fPCR *CMAH*	TTGAGCCATGCATTTCTGTC	ATTTAACATTTCCTTACCTGCAC	213	NM_001113015.1

Table 11. List of real-time PCR primers

Gene	Primer sequences (5'-3')		Size of PCR product (bp)	GenBank accession no.
	Forward	Reverse		
Bcl-xl	TGGTGGTTGACTTTCTCTCC	ATTGATGGCACTAGGGGTTT	134	AF216205
BAX	GCCGAAATGTTTGCTGACGG	CGAAGGAAGTCCAGCGTCCA	146	AJ606301
p53	CCTCACCATCATCACACTGG	GGCTTCTTCTTTTGCACTGG	213	NM_213824
p16	CTGGACACTTTGGTGGTCCT	GCGGGATCTTCTCCAGAGTT	185	AJ316067
***DNMT*1**	TCGAACCAAAACGGCAGTAG	CGGTCAGTTTGTGTTGGAGA	215	DQ060156.1
***DNMT*3a**	CTGAGAAGCCCAAGGTCAAG	CAGCAGATGGTGCAGTAGGA	238	NM_001097437.1
***DNMT*3b**	AGTGTGTGAGGAGTCCATTGCTGT	GCTTCCGCCAATCACCAAGTCAAA	133	NM_001162404.1
GLUT1	GCTTCCAGTATGTGGAGCAACT	AAGCAATCTCATCGAAGGTCC	132	X17058.1
LDHA	ATCTTGACCTATGTGGCTTGGA	TCTTCAGGGAGACACCAGCAA	214	NM_001172363.1
GAPDH	TCTCTGCTCCCTCCCCGTTC	TGGCAATGCACGGAACACAC	51	AF017079

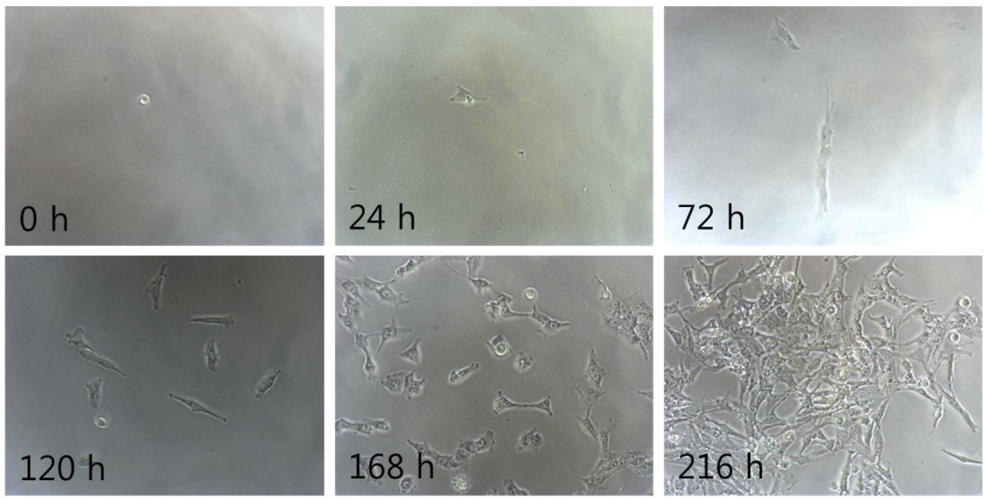


Figure 6. Single cell culture of immortalized cell. Single cell in 4 μl drop of culture media were observed by under left listed time. Only one cell was plated on the cell culture dish (0 h), after 24 h that cell attached onto the dish. Attached cell divided continuously with time to establish the one colony at 216 h.

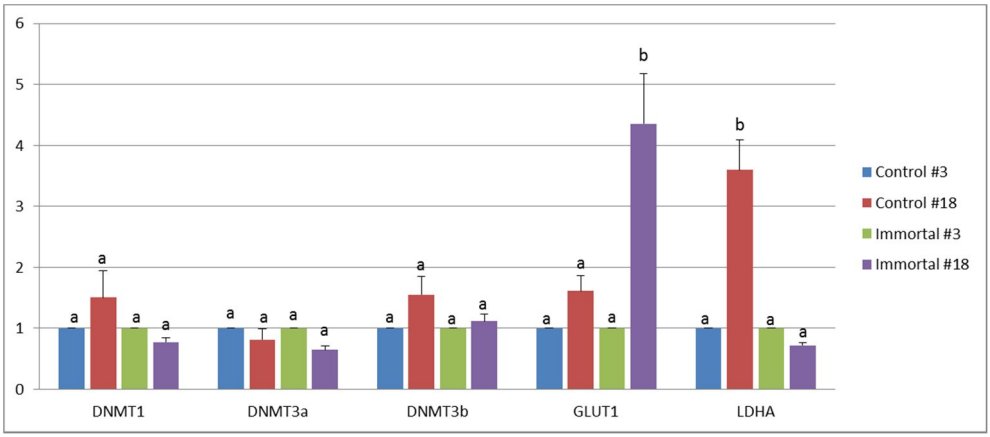


Figure 7. Comparison of methylation and metabolism related gene expression between control and immortalized cells. Methylation related *DNMT*1, *DNMT*3a, and *DNMT*3b genes were evaluated. No significant differences were observed in methylation patterns of control cell lines between immortalized cell lines at any passages. However, in analysis of metabolic genes, expression of *GLUT1* and *LDHA* were significantly up regulated in late passage of immortalized and control cells, respectively.

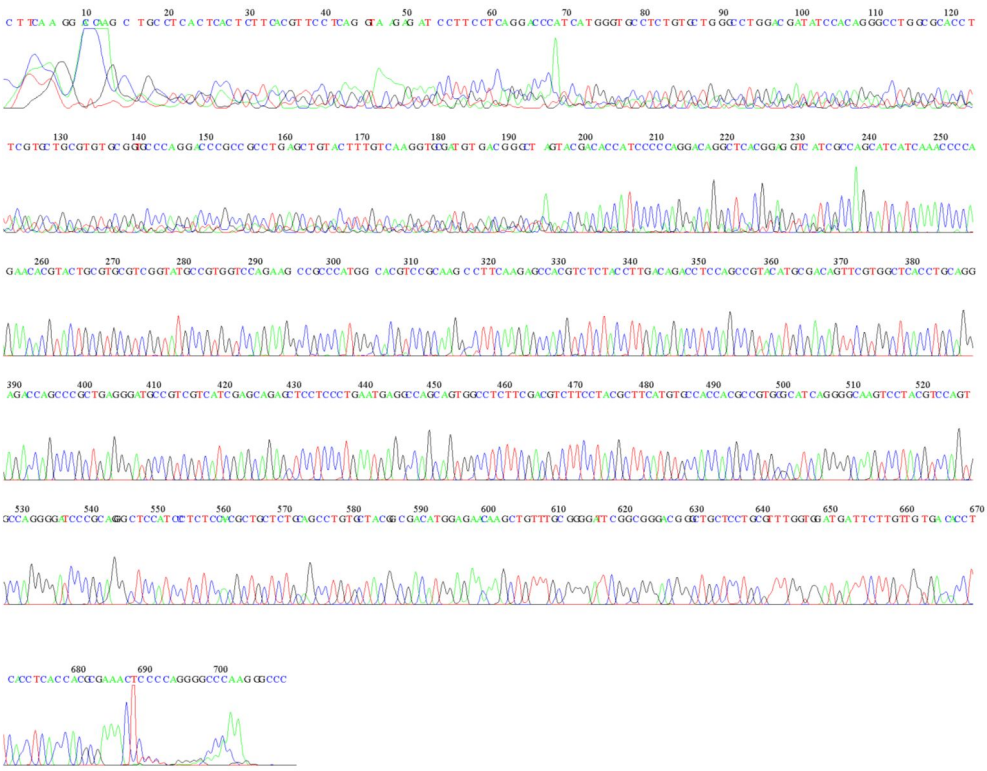


Figure 8. Sequencing results from genomic DNA from immortalized cell lines. These sequencing results were exactly same with the *hTERT* sequences.

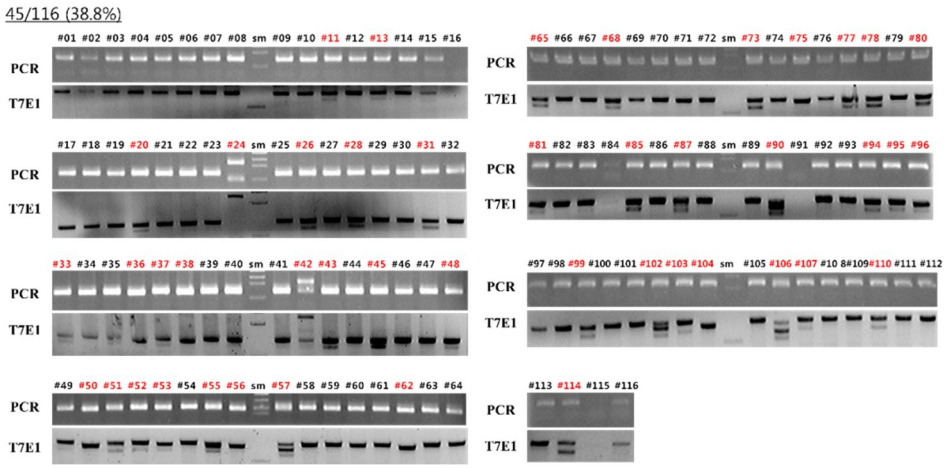


Figure 9. T7E1 assay results from *CMAH* KO single cell colonies. *CMAH* KO single cell colonies were subjected on T7E1 assay for confirming knocked out colonies. Among 116 colonies, 45 colonies were knocked out, which efficiency rate was 38.8%.

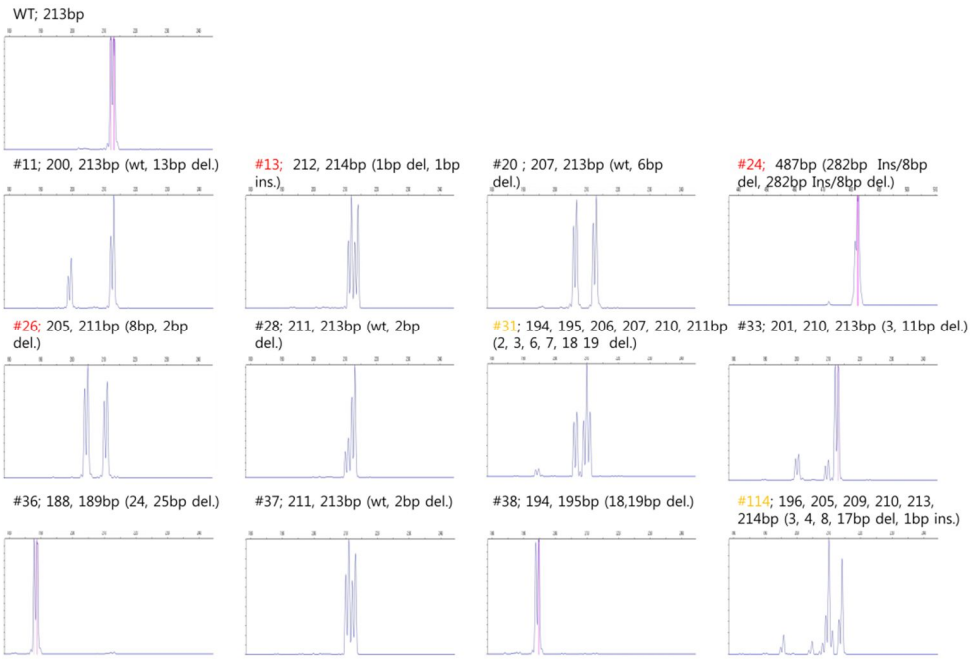


Figure 10. fPCR results from *CMAH* KO single cell colonies. Five colonies were confirmed that biallelic KO colonies with fPCR results (#13; 1bp deletion (del), 1bp insertion (ins), #24; 282bp ins/8bp del, 282bp ins/8bp del, #26; 8bp del, 2bp del, #36; 24bp del, 25bp del, and #38; 18bp del, 19bp del). Among them 3 colonies were strictly confirmed that biallelic KO colonies with fPCR and sequencing those were #13, #24 and #26 (red colored letter). Colony number 31 and 114 (yellow colored letter) were contaminated colonies. Other colonies (black colored letter) were monoallelic KO colonies.

AGTTCTAGCCACTAGACCACCAGGGAACTCCCTATTCTAAATT

CTTGAGCACATTATTTAGGAACCTCAGGAACTTGGCAAGGATT

ACAAGGAAATATATCTAGATTTAAAAAAAAATCTTTTAACAGAG

GTCCCAAAGGAGAGTCATGCACAGCTATGGGAGGAAGTTCAG

AAACTGCCCTTGCTACCAGATCACTGTCAGATAAAATGGCCAG

CTACATGTTTCTGCACATTGCCCTAAGATCTTTACAAACTTTTC

TGTGCATTTTTCCACTTTTAAAA

Figure 11. Inserted 282 bps sequence in #24 colony. Huge gene insertion was observed in #24 colony and hereof integrated gene sequences were quoted.

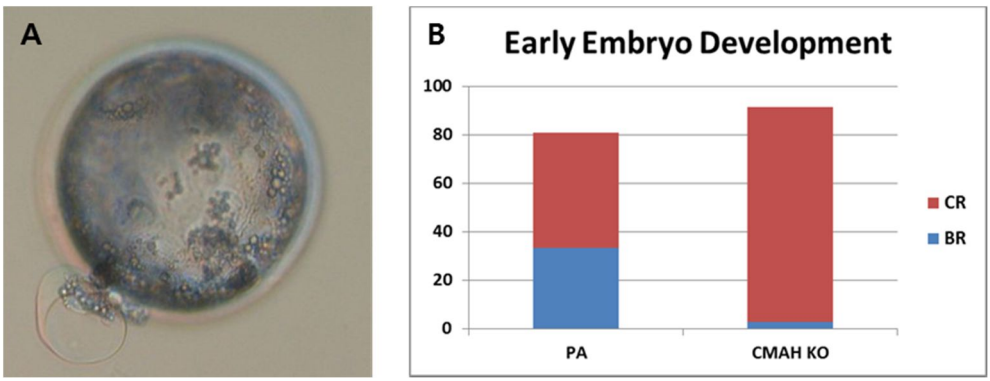


Figure 12. SCNT with *CMAH* KO donor cells. (A) A blastocyst derived from SCNT with *CMAH* KO donor cell. *CMAH* KO cells were employed into enucleated oocytes for determination of developmental competence. (B) Development rates were evaluated in two groups: parthenogenetic activated embryos (total numbers of oocytes: 69) and SCNT derived embryos using *CMAH* KO cells as nuclear donors (total numbers of oocytes: 36). Only one replicates were done in all two groups. CRs were 81.2% and 91.7%, respectively and BRs were 33.3% and 2.8%, respectively. (PA; parthenogenetic activation, KO; knockout, CR; cleavage rate, BR; blastocyst formation rate)

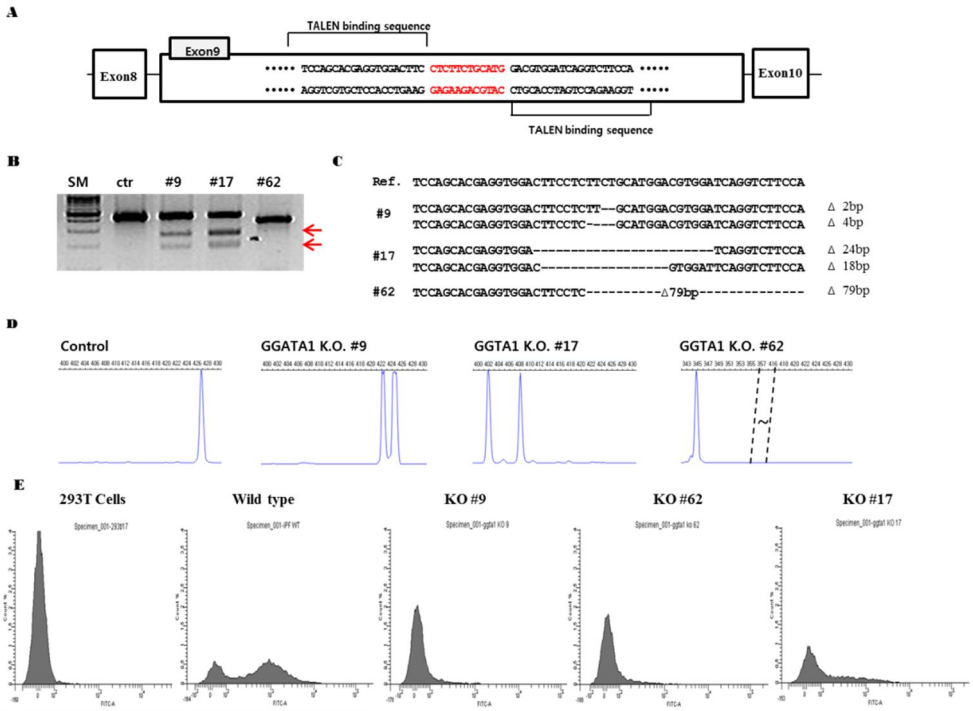


Figure 13. Illustration of TALEN binding sites and results of *GGTA1* TALEN KO. (A) DNA binding sequences and the spacer region for *GGTA1* TALEN. (B) T7E1 assays. (C) DNA sequences of the *GGTA1* locus from each *GGTA1* KO clones. (D) fPCR assay of the *GGTA1* KO clones. (E) FACS analysis of *GGTA1* KO clones.

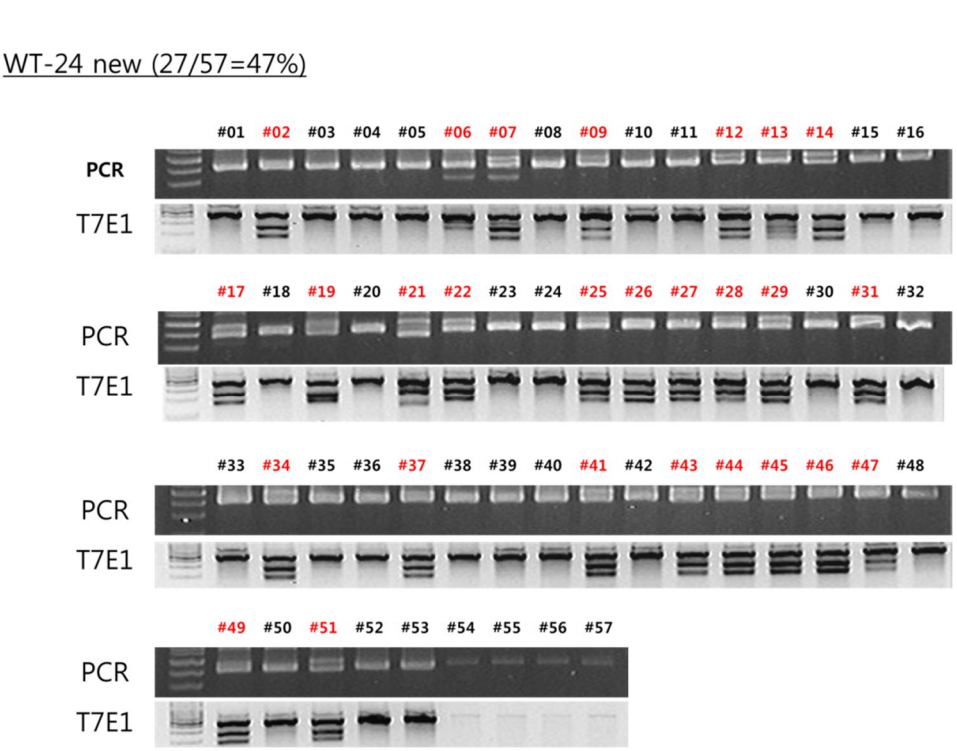


Figure 14. T7E1 assay results from 1st *GGTA1* KO single cell colonies. *GGTA1* KO single cell colonies were subjected on T7E1 assay for confirming knocked out colonies. Among 57 colonies, 27 colonies were knocked out, which efficiency rate was 47%.

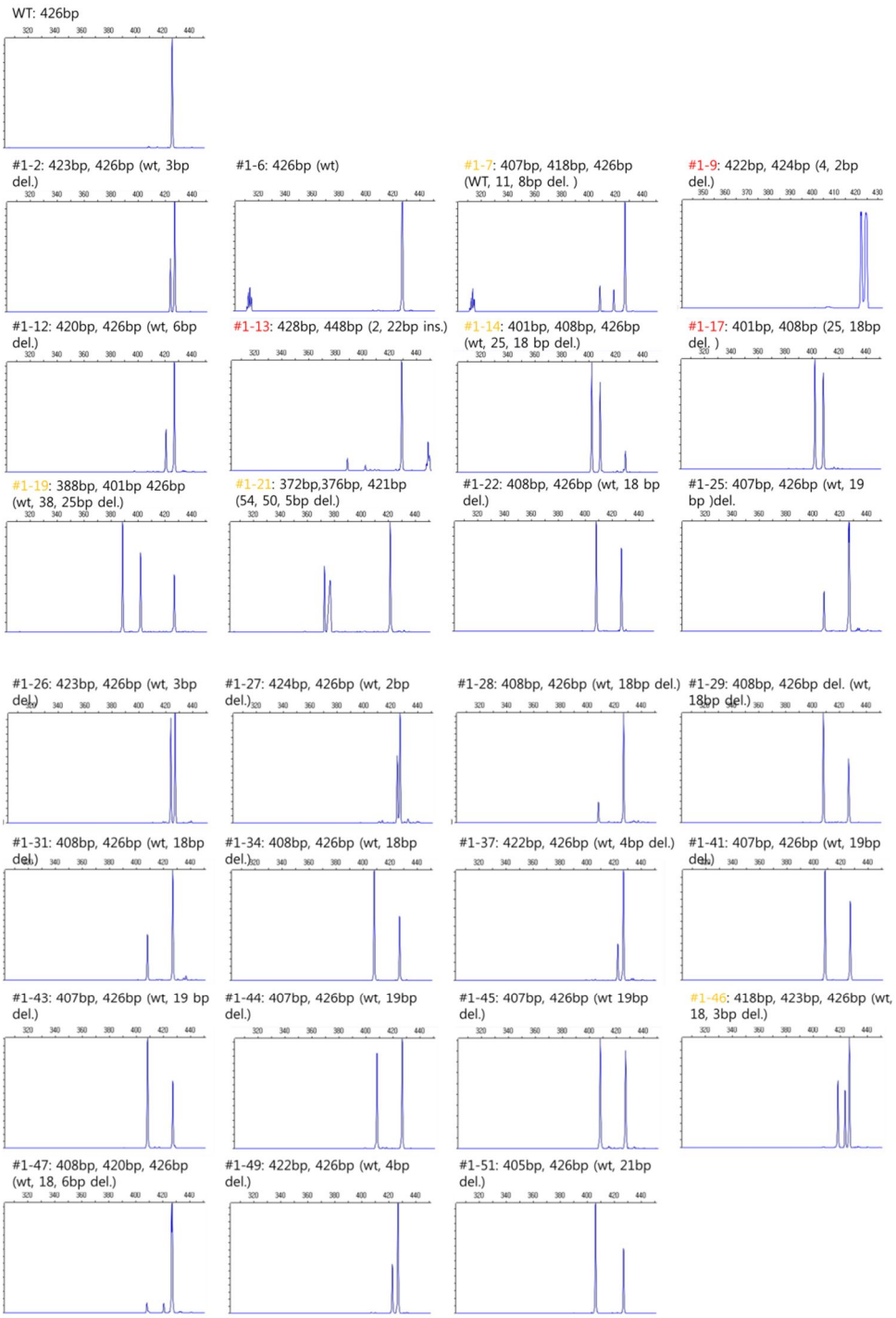


Figure 15. fPCR results from 1st *GGTA1* KO single cell colonies. Three colonies were confirmed that biallelic KO colonies with fPCR results (#1-9;

4bp del, 2bp del, #1-13; 2bp ins, 22bp ins, #1-17; 25bp del, 18bp del; red colored letter). Colony number 1-7, 1-14, 1-19, and 1-21 (yellow colored letter) were contaminated colonies. Other colonies (black colored letter) were monoallelic KO colonies.

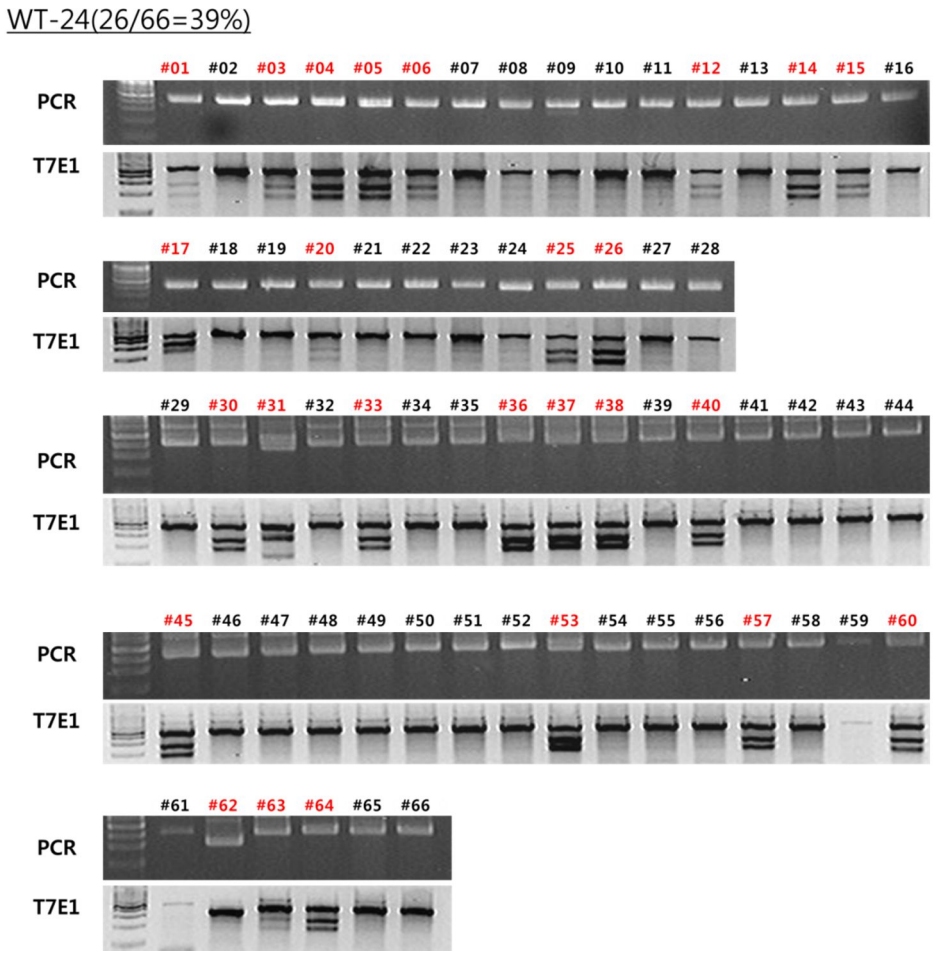


Figure 16. T7E1 assay results from 2nd *GGTA1* KO single cell colonies. *GGTA1* KO single cell colonies were subjected on T7E1 assay for confirming knocked out colonies. Among 66 colonies, 26 colonies were knocked out, which efficiency rate was 39%.

Figure 17. fPCR results from 2nd *GGTA1* KO single cell colonies. Two colonies were confirmed that biallelic KO colonies with fPCR results (#2-31;

3bp del, 53bp del, #2-62; 72bp del, 72bp del; red colored letter). Colony number 2-3, 2-4, 2-5, 2-6, 2-12, 2-14, 2-15, 2-26, and 2-60 were contaminated colonies. Other colonies were monoallelic KO colonies.

4. Discussion

It is well established in humans and in mice that cell lines are necessary to understand or to evaluate the molecular process (Todaro and Green, 1963; Graham *et al.*, 1977; Scherer *et al.*, 1953). However, in the pig, such research has been limited to date. In this study, I developed immortalized cells and furthermore those cells were used for TALEN to KO the interesting genes.

For inducing immortalization, genes such as *SV40 large T antigen*, m*BMI* and *hTERT* were used in previous studies (Garcia-Escudero *et al.*, 2010). Among these genes, *hTERT* has especially been used to immortalize cells because of reduced chromosome damage (Ray *et al.*, 1990; Garcia-Escudero *et al.*, 2010). In this study, *hTERT* successfully induced pig fetal fibroblasts into immortalization. In addition, our immortalized cells can be cultured for single cell colony formation at least three times. Therefore, single immortalized cell was cultured and propagated to unlimited numbers, indicating that single mutated cells can be isolated and used for many assays requiring cells, DNAs, RNAs and proteins.

For investigating properties of the immortalized cells, expression of proliferative, apoptotic, metabolic and methylation related genes were analyzed. Even though significant change of *p53* expression at specific passage was observed during long term culture, *p16* was dramatically down regulated (Figure 3D) from passage 6. The fact that loss of *p16* function

after transfection with *hTERT* is much related to immortalization is on the same line with our results (Kim *et al.*, 2002). As expected, antiapoptotic gene (*Bax*) was increased but proapoptotic gene (*Bcl-xl*) was decreased. In metabolic gene expression, *GLUT1* was significantly increased because immortalized cell utilize much more glucose for unlimited cell proliferative competence like cancer cell (Macheda *et al.*, 2005). And expression of *LDHA*, which is soluble cytosolic enzyme resulting from apoptosis or necrosis was increased in late passage control cells (Chan *et al.*, 2013). One point is about methylation gene expression. In contrast to our results, a previous study reported increase of *DNMT*1 expression in human fibroblast after *hTERT* transfection (Young *et al.*, 2003). However, in our case, the *DNMT*s expression levels were not changed after immortalization. In this study, I found that down regulation (*p16* and *Bax*) and up regulation (*GLUT1* and telomerase activity) plays an important role in maintaining the unlimited cell propagation (Figure 3D, Figure 5, and Figure 7).

Additionally, it has raised a scientific interest on SCNT embryo production using immortalized cells with long term culture properties. With respect to early embryonic development, no significant differences were observed in CRs among PA embryos, SCNT embryos derived with control cells and SCNT embryos derived with immortalized cells. However, significant differences were observed in BRs among these three groups. In particular, BRs were significantly decreased in the immortalized cell SCNT group. The

very low embryonic development using immortalized cells is similar to that reported in a previous study, in which immortalized bovine epithelial cells used in SCNT could not support embryo development into blastocysts (Zakhartchenko *et al.*, 1999). However, with ovine immortalized fibroblasts using *hTERT* as in this study, there was no significant difference between control and immortalized groups in their ability to support SCNT early embryo development (Cui *et al.*, 2003). In two previous studies, (Cui *et al.*, 2003; Zakhartchenko *et al.*, 1999), it was concluded that transformed cells with abnormal cellular responses (like serum starvation) failed to support embryonic development into blastocysts. Therefore, I assume that morphological and proliferative changes in our cells immortalized using *hTERT* affected normal cellular gene expression profiles including *p16* and resulted in very low embryonic development. This is another possible reason why SCNT embryos using immortalized cells could not well developed to blastocysts.

TALEN, an emerging genome editing tool can be applied to generate mutant pigs. To KO a gene using TALEN, several pairs on a specific coding domain region should be designed and evaluated for choosing the most effective pairs. Thus, effective TALEN DNA pairs deleted the DNA with an efficiency of 3.9% - 43% to date (Kim *et al.*, 2013b). However, validation systems to determine effective pairs in different species could provide different genome editing efficiencies (Yang *et al.*, 2011). In fibroblasts,

before homogeneous KO cell lines were achieved, single isolated mutated cells became senescent and thus could not be subjected to further analysis and application. Therefore, I strongly suggest that in porcine genome editing, these immortalized cells could be used as appropriate *in vitro* test cell lines to select effective pairs of TALEN.

Because in this study our immortalized cell line can be grown up from a single cell into billions of cells, I randomly chose one hundred single cells and cultured them into colonies of homogeneous s cells. Using single cell colony formation competence as a selection criterion, I can more easily generate KO cell lines. Indeed, in this study, single cell colonies grew well and these were used to analyze each colony to evaluate mutation characteristics. As a result, TALEN activity showed 38.8% efficiency and many cell lines were isolated (Figure 9). From the finally chosen cells, three biallelic mutated cell lines developed that did not express the cell surface carbohydrate chain, *Neu5Gc*. From one of these three mutated cell lines, colony number 24, cells were used as nuclear donor cells for SCNT. Although the BR was low, I observed that immortalized cells with *CMAH* KO can be reprogrammed in porcine enucleated oocytes and develop to the blastocyst stage. Additionally, the other gene, *GGTA1*, which is responsible for hyperacute rejection in xenotransplantation, was also knocked out in this cell line using exactly the same methods with high efficiency (Figure 13-17) (Kwon *et al.*, 2013).

In conclusion, the *hTERT* gene prolonged the usual life span of porcine fibroblasts into immortalized status. Immortalized cells with single cell survival properties were treated with TALE nucleases to delete *CMAH*. Then knocked out cells were employed to generate preimplantation embryos. These immortalized cells must become useful tools as an *in vitro* model to select the most effective TALEN pairs and KO specific genes to support development of biomedically useful pig models.

PART IV

PRODUCTION OF GENETIC MODIFIED PIGS

Chapter I. Transgenic pigs overexpressing human *SNCA*.

1. Introduction

Among many chronic and progressive diseases afflicting humans, PD is the most common neurodegenerative movement disorder (Lees *et al.*, 2009), that can affect 1% of people over age 60 (Dawson *et al.*, 2010) and 4-5% of people over age 85 (Dawson and Dawson, 2002). Bradykinesia, muscle rigidity, tremor at rest and disturbances in balance are major symptoms of PD (Bohlhalter and Kaegi, 2011; Lang and Lozano, 1998a; Lang and Lozano, 1998b). PD is commonly associated with destruction of dopaminergic neurons in the substantia nigra pars compacta and is characterized by the presence of Lewy bodies in the intracytoplasmic regions (Yue and Lachenmayer, 2011). Genes that are related to PD are *SNCA* and *LRRK2*, autosomal dominant PD, *PINK1*, *PRKN*, and *DJ-1*, autosomal recessive PD (Blesa *et al.*, 2012). The etiology of PD is unclear, however, the most common association of PD is an accumulation of aggregated SNCA in specific brain stem, spinal cord and cortical regions (Lees *et al.*, 2009; Hawkes *et al.*, 2010).

Among those PD related genes, human *SNCA*, which is translated into a 140 amino acid protein, exists naturally in an unfolded form. Nevertheless, folded forms of SNCA are frequently observed in Lewy bodies, the

hallmarks of neurodegenerative diseases such as PD (Brandt *et al.*, 2008; Fearnley and Lees, 1991; Hawkes *et al.*, 2010).

Transgenic models for PD have been produced with overexpression of *SNCA* in many species, such as drosophila (Dawson *et al.*, 2010), mice (Oliveras-Salva *et al.*, 2013; Dawson *et al.*, 2010), rats (Gombash *et al.*, 2013; Dawson *et al.*, 2010) and monkeys (Eslamboli *et al.*, 2007). However, the need for physiological similarity with humans as well as economic reasons necessitate development of alternative models for PD such as pigs (Glud *et al.*, 2011; Swindle *et al.*, 2012).

Therefore, pigs were selected as the model animals in this study, and *SNCA* genes were randomly inserted into Yucatan kidney cells for introduction into oocytes of pigs *via* SCNT. The SCNT oocytes were then activated and cultured, then transferred into oviducts of surrogate pigs to produce offspring (Koo *et al.*, 2009). The cloned pigs were analyzed by genetic and behavioral methods to verify similarities with human PD patients.

2. Materials and Methods

2.1. Primary cell culture

Adult female Yucatan kidney (16 months old) cells, which were used as control cells, were isolated and cultured. Kidneys were washed 3 times in PBS and then following the protocol previously established in our studies (Moon *et al.*, 2014a). In brief, the washed kidney was chopped into small pieces with trypsin. Trypsinized tissues were then incubated for 30 min at 37^{o}C. Well dissociated tissues were centrifuged at 1,500 rpm for 2 min. The supernatant was discarded and the pellet was resuspended with PBS then centrifuged at 1,500 rpm for 2 min. These procedures were repeated 2 times. Finally, the supernatant was discarded and the pellet was resuspended in DMEM (Gibco, Carlsbad, California, USA) supplemented with 15% FBS (Gibco), 1% P/S (Gibco), 1% NEAA (Gibco) and 100 mM β-ME by inverting the tube several times. The cells resuspended in this medium were held at room temperature (around 25^{o}C) for 5 min, then the suspension was transferred into a cell culture dish and cultured approximately 10 days with culture medium changes every 2-3 days. These primary cells were cultured, expanded and frozen at -196^{o}C for further use. The cell cultures were maintained in DMEM with 15% FBS, 1% P/S, 1% NEAA and 100 mM β-ME.

2.2. Construction of retrovirus vector and preparation of transfected

cells

A plasmid, p*LVC-SNCA-eGFP*, containing a full-length human *SNCA*, was transfected into the experimental cells by viral infection (Supplementary Figure 1). In brief, the lentiviral vector was generated by co-transfection of Lenti X-293T (Clontech Laboratories, Inc., Otsu, Shiga, Japan; catalog no. 632180) cells with Lenti X-HTX packaging mix (Clontech, catalog no. 631260) using the calcium phosphate method. Virus particles were harvested 48 h post transfection. Virus producing cells were grown at 37oC, in a 5% CO2 incubator in high glucose DMEM supplemented with 10% tetracycline free fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), 0.1 mM MEM NEAA, 6 mM L-glutamine, and 1 mM MEM sodium pyruvate. The virus containing medium harvested from the virus producing cells was filtered through a 0.45 μm pore size filter before applying it to the Yucatan kidney cells for infection. After 16 h of incubation, the culture medium was changed and the cells were incubated for 2 additional days. Cells expressing *eGFP* were identified using fluorescence microscopy.

2.3. PCR

Genomic DNA was extracted with the G-spin Genomic DNA Extraction Kit (iNtRON) according to the manufacturer's protocol. Amplification of target genes was achieved using Maxime PCR PreMix (i-StarTaq, iNtRON).

Primer sets, conditions and expected sizes are annotated in Table 12.

2.4. RT-PCR

Total RNAs were extracted to analyze gene expression in the immortalized cells by using the easy-spin Total RNA Extraction Kit (iNtRON). Then, cDNAs were synthesized using Maxime RT Premix (iNtRON) according to the manufacturer's protocol.

Information on primers is listed in Table 12. Gene expression for *SNCA* was measured.

2.5. SCNT and delivery of cloned piglets

Transfected donor cells were subjected to SCNT, which was done by following the protocol established in our previous study (Koo *et al.*, 2009). Briefly, immature oocytes were obtained from pig ovaries by aspiration of follicles and cultured for 40 h to support maturation. The *in vitro* matured (IVM) oocytes were enucleated using an aspiration pipette, then microinjected with an *SNCA* overexpressed donor cell, fused by electrical stimulation and activated using an electrical protocol. On day 2, reconstructed embryos were surgically transferred into the oviducts of recipient females. Pregnancy status was determined by ultrasonography on day 28 after ET. Cloned piglets were delivered by caesarean section and their clonal status was confirmed by PCR and RT-PCR based assays.

2.6. ELISA analysis

Blood samples were collected from the jugular veins of 4 month and 1 year control pigs with the same ages as the cloned pigs. Proteins were collected using PRO-PREP^{TM} for Cell / Tissue Protein Extraction Solution (iNtRON biotechnology, Gyeonggido, Korea) according to the manufacturer's protocol. Expressions of *SNCA* in 4 cloned pigs were evaluated with the α-Synuclein Human ELISA Kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol.

2.7. Analysis of *SNCA* pigs using a motor scoring system

The scoring analysis table for cloned pigs was based on UPDRS. The UPDRS is most broadly accepted clinical scale for the evaluation of PD patients (Ramaker *et al.*, 2002). Motor scores of cloned pigs were accomplished 2 times (at 5 months of age and 1 year) by following our previously established protocol (Moon *et al.*, 2014b).

2.8. Statistical analysis

All data were analyzed by one way ANOVA followed by Tukey's Multiple Comparison Test using GraphPad Prism version 5.01 (GraphPad Software, Inc., San Diego, California, USA) to determine differences among experimental groups. Statistical significance was determined when the *p*-value was less than 0.05.

3. Results

3.1. Production of *SNCA* expressing cells and blastocysts

A plasmid, p*LVC-SNCA-eGFP*, was constructed and transfected into cells *via* lentiviral infection (Figure 18). Before transfection, no cells expressed *eGFP* signals under UV. However, transfected cells that were selected by neomycin treatment showed *eGFP* expression (Figure 19B and B'). To confirm the suitability of donor cells for SCNT, transfected cells were inserted into oocytes and activated for development into blastocysts (Figure 19C). Signals for *eGFP* were detected in fully developed blastocysts (Figure 19C').

Insertion of *SNCA*, which is 194bp, in donor cells was identified by PCR (Figure 20A) and transcription of *eGFP* in donor cells was also confirmed by RT-PCR. In Figure 20B, the upper line indicates *eGFP* bands and the lower line indicates *GAPDH* bands. Both data ascertained that *SNCA* genes were well integrated into genomic DNAs and inserted genes were properly transcribed into messenger RNAs. Furthermore, transcription of *SNCA* in blastocysts was confirmed by RT-PCR (Figure 20C).

3.2. Production of *SNCA* expressing piglets and its characteristics

Among 23 offspring, only 4 remain alive which are designated as PDF4, PDF16, PDF18 and PDF20 (the survival rate was 17.4% in this experiment.). Piglet PDF4 was born by caesarean section while the other 3 piglets were

born by normal full term delivery. Most of the dead piglets expressed *eGFP* under UV (data not shown). In piglet PDF2, strong expression of *eGFP* was detected in the whole body, intestine, heart, lung and brain. However, the expression level of *eGFP* was weak in spleen, liver, and kidney (Figure 21).

Moreover, genomic DNA integration of *SNCA* genes and their transcription were confirmed by PCR and RT-PCR. Among 23 piglets, it was confirmed in only one, PDF11, that the *SNCA* gene was not integrated into the genomic DNA. Nevertheless, while in 22 piglets existence of the *SNCA* gene was confirmed in genomic DNA, the *SNCA* gene was transcribed in only 11 piglets (PDF1, PDF2, PDF4, PDF6, PDF7, PDF16, PDF17, PDF18, PDF20, PDF21 and PDF22). Fortunately, four piglets (PDF4, PDF16, PDF18 and PDF20) that survived were identified as transgenic animals (Figure 25).

3.3. Analysis of ELISA and motor scoring using *SNCA* expressing piglets

Over 1 year, ELISA was performed twice. At 4 months of age, SNCA concentrations in blood were measured: PDF4 (0.46 ± 0.01 ng/ml), PDF16 (-0.06 ± 0.02 ng/ml), PDF18 (2.16 ± 0.04 ng/ml) and PDF20 (-0.04 ± 0.01 ng/ml). Only PDF4 and PDF20 showed significant differences ($p<0.05$) when compared to control (Figure 22). However, SNCA concentrations in blood changed at 1 year of age: PDF4 (10.90 ± 0.17 ng/ml), PDF16 (0.23 ±

0.17 ng/ml), PDF18 (-0.13 ± 0.01 ng/ml) and PDF20 (5.75 ± 0.14 ng/ml). The SNCA blood concentrations of PDF4 and PDF20 significantly increased during year 1 after birth. However, SNCA concentrations in the blood of PDF18 significantly decreased during the first year after birth, while that of PDF16 did not significantly change.

According to the scoring analysis, control pigs showed no change in score during the time points examined. The score for PDF18 decreased from '9' to '8'. However, after 7 months, scores increased from PDF4 (from '9' to '13'), PDF16 (from '10' to '11') and PDF20 (from '6' to '9').

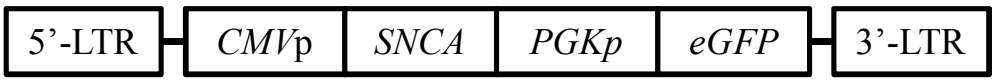


Figure 18. A schematic representation of p*LVC-SNCA-eGFP*.

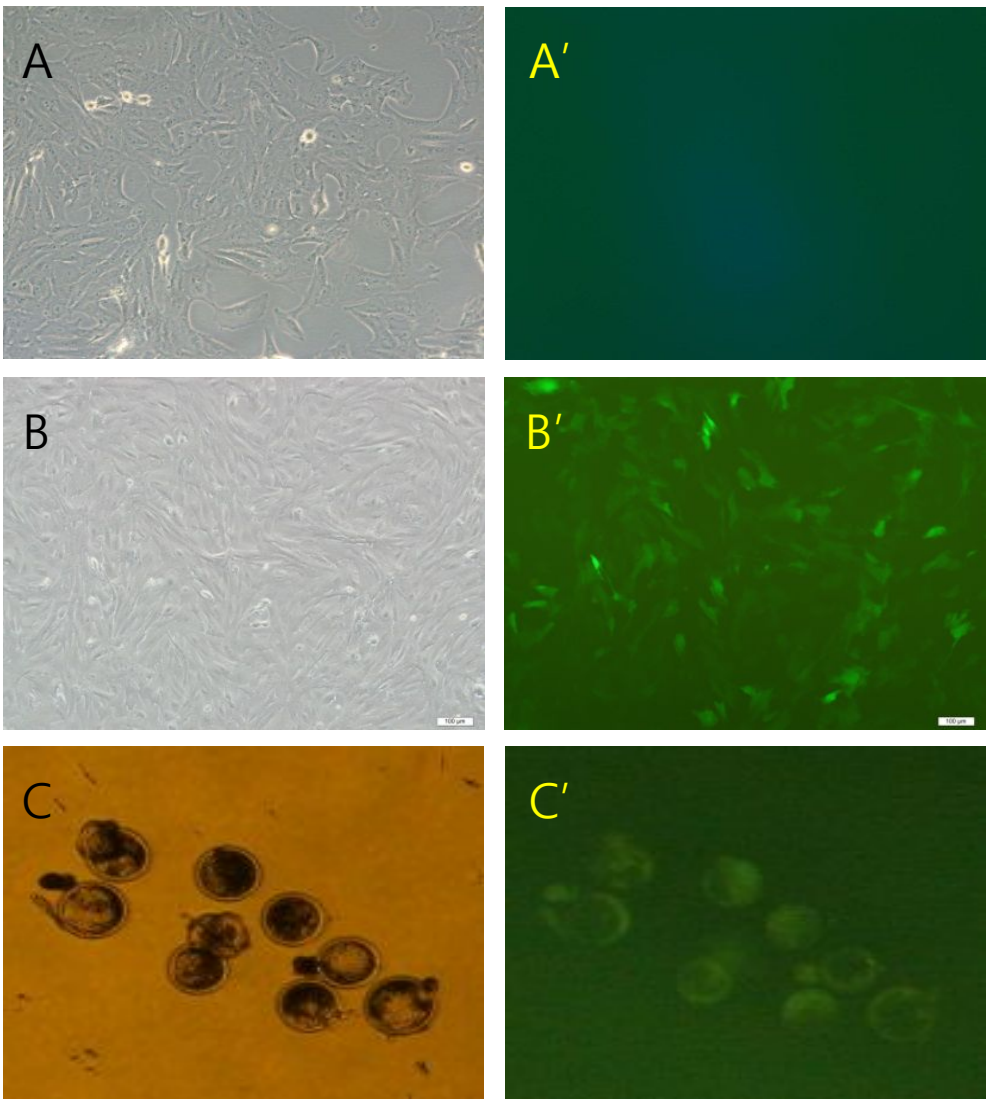


Figure 19. *SNCA* expression cells and blastocysts. (A and A') Normal Yucatan kidney cell line. No expression of *eGFP* was detected. (B and B') After transfection of p*LVC-SNCA-eGFP* in Yucatan kidney cells. *eGFP* expression was confirmed under UV. (C and C') Blastocysts derived from p*LVC-SNCA-eGFP* transfected cells. *eGFP* expression was also established in developing blastocysts.

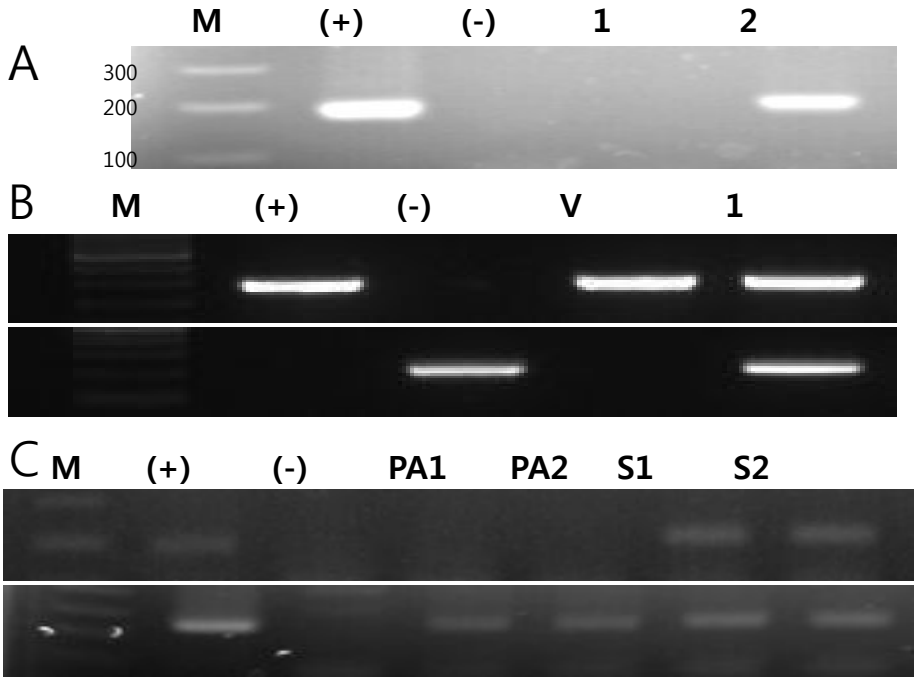


Figure 20. PCR and RT-PCR results for transfected cells and blastocysts. (A) Insertion of *SNCA*, which is 194bp, in donor cells identified by PCR (M; Marker, (+); Positive plasmid control, (-); Negative control, 1; Non-transfected Yucatan kidney cells, 2; *SNCA* transfected Yucatan kidney cells). (B) Transcription of *eGFP* in donor cells was also confirmed by RT-PCR. Upper line indicates *eGFP* bands and lower line indicates *GAPDH* bands (M; Marker, (+); Positive plasmid control, (-); Non-transfected Yucatan kidney cells, V/R; p*LVC-SNCA-eGFP* viral RNA, 2; *SNCA* transfected Yucatan kidney cells). Both data ascertained that *SNCA* genes were well integrated into genomic DNAs and inserted genes were properly transcribed into messenger RNAs. (C) Transcription of *SNCA* in blastocysts was confirmed by RT-PCR (M; Marker, (+); Positive plasmid control, (-);

Negative control, PA1; 8 Parthenogenetic activated blastocysts, PA2; another 9 Parthenogenetic activated blastocysts, S1; 4 *SNCA* transfected blastocysts, S2; another 4 *SNCA* transfected blastocysts).

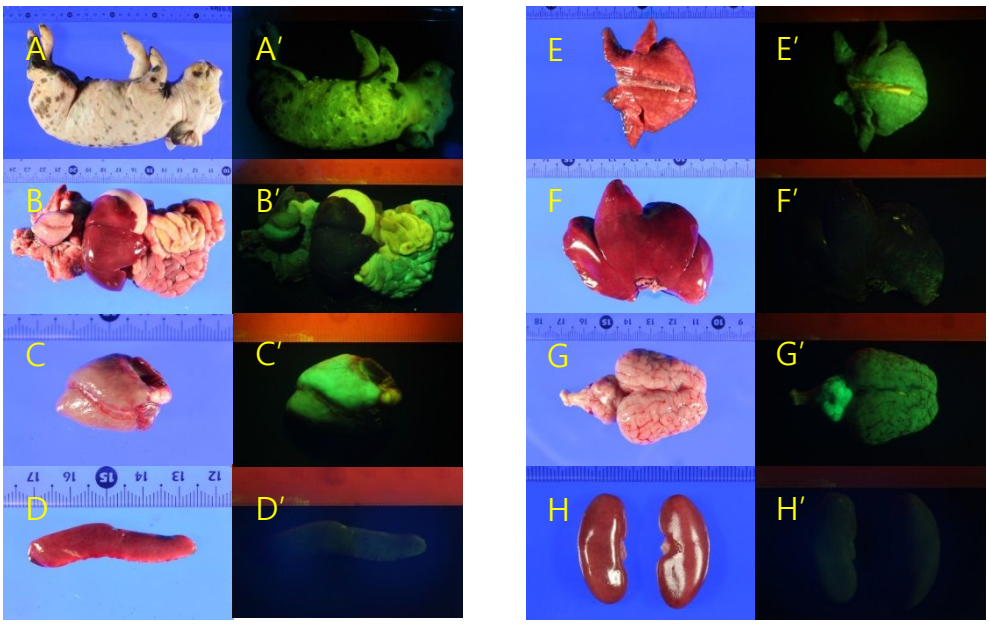


Figure 21. Expression of *eGFP* in PDF2. Among 23 newly born piglets, 19 died. All of the dead piglets were delivered to the laboratory for autopsy. Most of the dead piglets expressed *eGFP* under UV (data not shown). In PDF2, *eGFP* expression was highly detected in the whole body (A and A'), intestine (B and B'), heart (C and C'), lung (E and E'), and brain (G and G'). However, in spleen (D and D'), liver (F and F'), and kidney (H and H') the expression level of *eGFP* was weak.

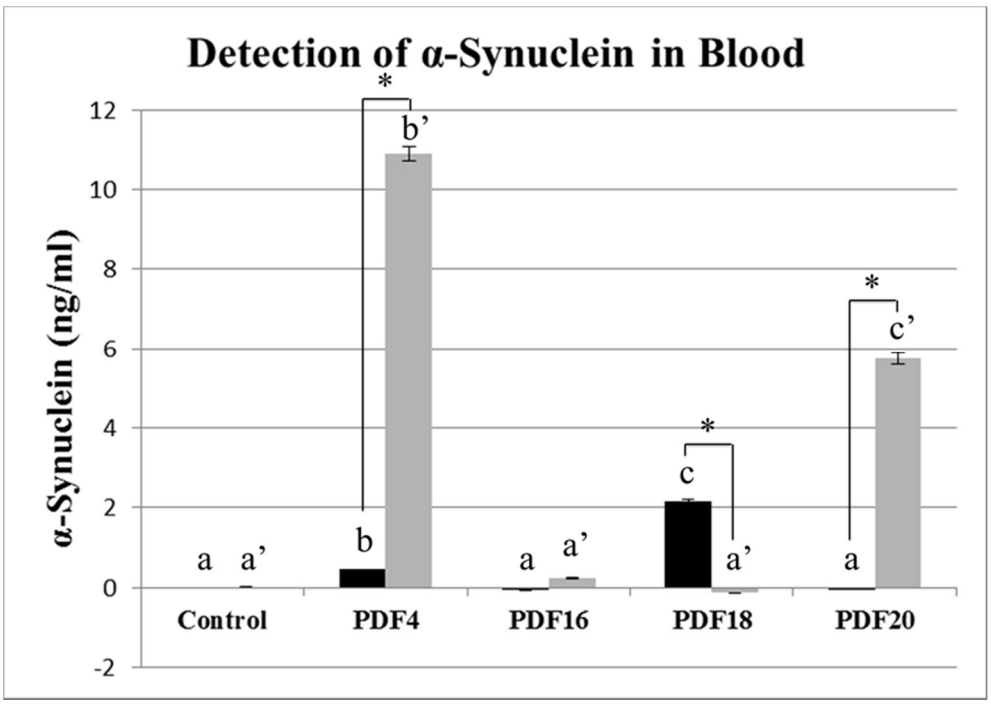


Figure 22. Detection of SNCA with ELISA in 4 month old (black bars) and 1 year old pigs (grey bars). At 4 months old, SNCA concentrations in blood were measured; PDF4 (0.46 ± 0.01 ng/ml), PDF16 (-0.06 ± 0.02 ng/ml) PDF18 (2.16 ± 0.04 ng/ml), and PDF20 (-0.04 ± 0.01 ng/ml). Only PDF4 and PDF20 showed significant differences ($p<0.05$) compared with control. However, SNCA concentrations in blood were altered at 1 year old; PDF4 (10.90 ± 0.17 ng/ml), PDF16 (0.23 ± 0.17 ng/ml), PDF18 (-0.13 ± 0.01 ng/ml), and PDF20 (5.75 ± 0.14 ng/ml). Using ELISA in 1 year old pigs, PDF4 and PDF20 showed significant differences ($p<0.05$) with control. Blood SNCA concentrations of PDF4 and PDF20 were significantly increased at 1 year after birth. However, blood SNCA concentrations of

PDF18 were significantly decreased 1 year after birth, while that of PDF16 was not changed.

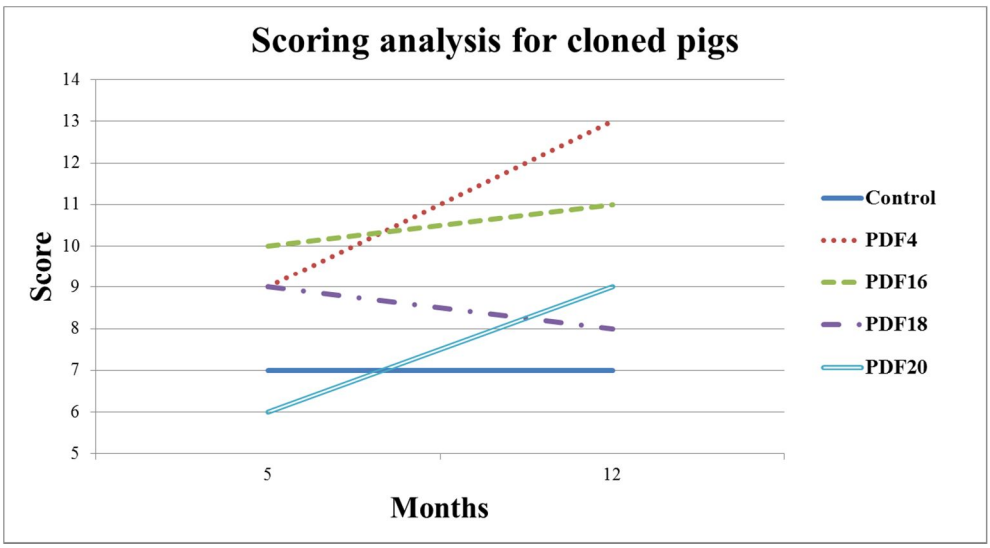


Figure 23. Scoring analysis for *SNCA* overexpression pigs. According to the scoring analysis for cloned pigs, control pigs showed unchanged scores over time. The score of PDF18 decreased from '9' to '8'. However, scores of PDF4 (from '9' to '13'), PDF16 (from '10' to '11'), and PDF20 (from '6' to '9') all increased with time.

ATGGATGTATTCATGAAAGGACTTTCAAAGGCCAAGGAGGGA

GTTGTGGCTGCTGCTGAGAAAACCAAACAGGGTGTGGCAGA

AGCAGCAGGAAAGACAAAAGAGGGTGTTCTCTATGTAGGCTC

CAAAACCAAGGAGGGAGTGGTGCATGGTGTGGCAACAGTGG

CTGAGAAGACCAAAGAGCAAGTGACAAATGTTGGAGGAGCA

GTGGTGACGGGTGTGACAGCAGTAGCCCAGAAGACAGTGGA

GGGAGCAGGGAGCATTGCAGCAGCCACTGGCTTTGTCAAAAA

GGACCAGTTGGGCAAGAATGAAGAAGGAGCCCCACAGGAAG

GAATTCTGGAAGATATGCCTGTGGATCCTGACAATGAGGCTTA

TGAAATGCCTTCTGAGGAAGGGTATCAAGACTACGAACCTGA

AGCCTAA

Figure 24. Sequence of the human *SNCA* gene.

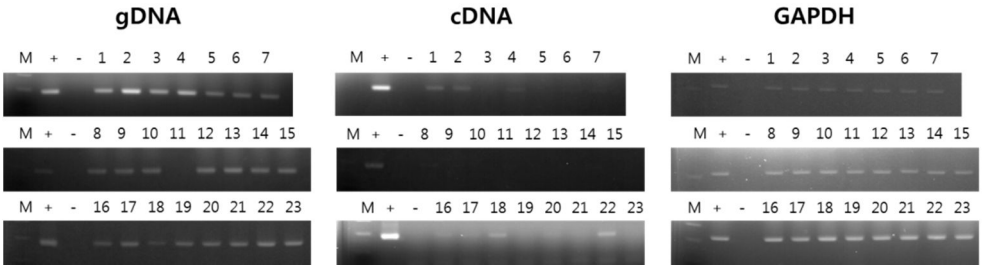


Figure 25. Detections of *SNCA* gene insertion and transcription. Genomic DNA integration of *SNCA* genes and their transcription were confirmed by PCR and RT-PCR, respectively, with tail samples. Among 23 piglets, it was confirmed in only one that the *SNCA* gene was not integrated into genomic DNA. Nevertheless, in 22 piglets it was confirmed that the *SNCA* gene was incorporated into the genomic DNA. Transcription of the *SNCA* gene was observed only in 11 piglets (PDF1, PDF2, PDF4, PDF6, PDF7, PDF16, PDF17, PDF18, PDF20, PDF21 and PDF22). Fortunately, four surviving piglets, PDF4, PDF16, PDF18 and PDF20, were identified as transgenic animals.

Table 12. Details of primers used for PCR and RT-PCR.

Gene	Primer sequences (5'-3')		Size of PCR product (bp)	GenBank accession no.
	Forward	Reverse		
SNCA	CTGAGAAAACCAAACAGGGTGT	CTCCACTGTCTTCTGGGCTACT	194	AY049786
eGFP	GACTTCAAGGAGGACGGCAACA	TCTCGTTGGGGTCTTTGCTCAG	256	U55762.1
GAPDH	TCGGAGTGAACGGATTTG	CCTGGAAGATGGTGATGG	219	AF017079

4. Discussion

Transgenic animal PD models have been produced in many species including drosophila (Dawson *et al.*, 2010), mice (Oliveras-Salva *et al.*, 2013; Dawson *et al.*, 2010), rats (Gombash *et al.*, 2013; Dawson *et al.*, 2010) and monkeys (Eslamboli *et al.*, 2007). However, no other PD model showed exactly the same disease progression as human PD patients. Because pathophysiological similarities exist between pigs and humans (Swindle *et al.*, 2012; Glud *et al.*, 2011), similar phenomena could be invoked after producing *SNCA* overexpression in pigs. The CMV promoter is a strong promoter which is mostly expressed in mammalian cells. Thus, our study is the first to produce pigs overexpressing the *SNCA* gene under the control of the CMV promoter.

I produced 4 pigs *via* SCNT overexpressing *SNCA*. These cloned pigs were confirmed by PCR and RT-PCR, and I mainly focused on clinical signs in these pigs. Most of these cloned animals were asymptomatic. First, protein translation was observed over time by SNCA concentration using ELISA. Additionally, behavioral abnormalities were monitored by scoring analysis.

Freichel *et al*. found that age dependent SNCA fibrillization in specific cortical regions was related to cognitive decline in SNCA transgenic mice (Freichel *et al.*, 2007). In transgenic pigs overexpressing *SNCA*, similar results were obtained by comparing ELISA results to motor scoring analysis. In piglets PDF4 and PDF20, SNCA concentrations in blood significantly

increased with age. Correspondingly, the motor scoring analysis values increased. Otherwise, in the case of PDF18, SNCA concentrations in blood significantly decreased with age. Also, motor scoring analysis values decreased. Piglet PDF16 showed little change in SNCA concentrations with age, and a similar pattern was observed in the motor scoring analysis. Thus, I found that blood SNCA concentration changes were tightly correlated with motor scoring behavioral changes. Previous studies revealed that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), neurotoxin, treated pigs showed abnormal behavior above a score of '11' (Moon *et al.*, 2014b). Piglets PDF4 and PDF16 had to be monitored intensively because their scores already exceeded '11'. Additionally, piglets PDF18 and PDF20 were confirmed by PCR base analysis as cloned pigs. These two pigs also needed to be monitored carefully for abnormal behavioral changes. Extrapolating to human early onset familial PD patients' ages, behavioral abnormalities can be detected several years later in pigs. Behavioral abnormalities and SNCA concentrations need to be continuously monitored for several years. Further studies using brain imaging systems; CT, MRI and PET-CT are planned.

In conclusion, the cloned pigs overexpressing *SNCA* were successfully produced by SCNT. Among 23 cloned pigs produced, insertion of *SNCA* into genomic DNA was confirmed in 4 live pigs, in which correct transcription to messenger RNA was observed. Two cloned pigs showed

elevation of SNCA concentrations and motor scoring analysis values with age.

Chapter II. *PRKN* KO pig production *via* TALEN.

1. Introduction

PD is commonly emerged with destructions of dopaminergic neurons in substantia nigra pars compacta (Yue and Lachenmayer, 2011). Numerous genes are related with early onset PD (Autosomal dominant genes; *SNCA, UCHL1*, and *LRRK2*, autosomal recessive genes; *PRKN*, *PINK1*, *DJ-1*, and *ATP13A2*) (Blesa *et al.*, 2012). In brief, autosomal dominant genes thought to cause PD, though autosomal recessive genes seemed to protect the neurons from toxic damages.

Among those genes, *PRKN* gene, which translated into PRKN protein, was chosen for KO pig production. PRKN is the component of the multi-protein complex, E3 ubiquitin ligase. The function of PRKN is unclear but, it degrades toxic substances to dopaminergic neurons leads to decrease of those concentrations in neurons. Thus mutation in PRKN allows increasing of toxic damages to mitochondria in neurons, followed by malfunction and death of neurons (Dawson *et al.*, 2010). Setting aside numerous causative factor modifications, only one protective gene KO was expected to asymptomatic pig production. Further cross breed with other gene modified pigs was important.

Several previous studies revealed that KO by HR in pigs was effortful (Lai *et al.*, 2002; Rogers *et al.*, 2008; Suzuki *et al.*, 2012). However, one of

emerging technologies, TALEN greatly improves KO efficiency (Xin *et al.*, 2013).

Eight KO piglets were produced by combination of SCNT and TALEN technologies. Among them 3 were confirmed as a KO cloned piglets. However those KO piglets were dead on the day of birth with several reasons (e.g. cleft, stillbirth, and unclear reasons). The KO cell lines established from those piglets except stillbirth one. Further trials are scheduled to produce the same gene KO healthy pigs.

2. Materials and Methods

2.1. Primary cell culture

Male Sinclair kidney cells, which were used as control cells, were isolated and cultured. Kidneys were washed 3 times in PBS and then following the protocol previously established in our studies (Moon *et al.*, 2014).

2.2. *PARK2* KO using TALE nucleases

Plasmids encoding TALENs that target exon of the pig *PRKN* gene and reporters for enrichment of TALEN mediated mutant cells used in this study were obtained from ToolGen Inc. (Seoul, Korea) (Kim *et al.*, 2013b). For delivery of plasmids, $5x10^5$ pig primary cells were electroporated using Neon device (Invitrogen) with a total of 5 μg DNA at a weight ratio of 2:2:1 (plasmid encoding a left TALEN: plasmid encoding right TALEN: flow cytometer reporter) or 4.5:4.5:1 (Kim *et al.*, 2011; Kim *et al.*, 2013a), respectively. Both transfected cells were separately cultured for two days at 37^oC. Then those were checked under the inverted microscope by morphologically; size and shape. Then, suitable cells were selected between two cells before every SCNT without any selection.

2.3. SCNT and delivery of KO piglets

Selected high performance TALEN pairs and reporters are transfected into the Sinclair male kidney cells every week and used for SCNT donor cells

without any selection processes (e.g., antibiotic, fluorescent, and magnetic based selections). In brief, reporter itself has own *RFP* and out framed *GFP*. Thus if reporters are properly transfected into cells, *RFP* signals were detected. Coincidently, TALEN pairs are integrated into the cells, out framed *GFP* is frame shifted then *GFP* signal started to emit 2 to 3 days after transfection from both TALEN and reporter transfected cells.

Only green fluorescence emitting cells were subjected to SCNT, which was done following the protocol previously established in our studies (Koo *et al.*, 2009). Briefly, immature oocytes were obtained from pig ovaries and cultured for 40 h to support maturation. The IVM oocytes were enucleated using an aspiration pipette, then microinjected with a control or transfected donor cell, fused by electrical stimulation and activated using an electrical protocol. On day 2, reconstructed embryos were surgically transferred into the recipient oviducts. The pregnancy condition was determined by ultrasonography on day 28 after ET. The cloned piglets were delivered by C-sec or normal childbirth.

2.4. T7E1 assay

After 48h of electroporation, genomic DNA was isolated from cells using Exgene^{TM} Cell SV mini kit (GeneAll Inc., Seoul, Korea). For validation of blastocysts, Blastocysts were pretreated with Direct PCR Lysis Reagent (Viagen Biotech Inc., California, USA) containing 0.4 mg/ml Proteinase K

at 55°C for 6h and then Proteinase K was inactivated at 85°C for 45min before the PCR reaction. Target locus was amplified using *PRKN* specific primer set and used for the T7E1 assay as previously described (Kim *et al.*, 2009). Briefly, the PCR amplicons were denatured and annealed to form heteroduplex DNA using a thermocycler and then digested with T7E1 (ToolGen Inc.) for 20 min at 37°C and then analyzed using agarose gel electrophoresis.

2.5. Sequencing

For sequencing analysis, PCR products corresponding to genomic modifications were purified and cloned into the T-Blunt vector (SolGent Inc., Daejeon, Korea). Target DNA samples were delivered to a sequencing company (Macrogen Ltd., Seoul, Korea). Briefly, sequencing reactions were performed in the DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD) using the ABI BigDye (R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using a M13 primer. The fluorescent labeled fragments were purified by the method recommended by Applied Biosystems because it removes unincorporated terminators and dNTPs. The samples were subjected to electrophoresis in an ABI 3730xl DNA Analyzer (Applied Biosystems).

3. Results

3.1. Production of *PRKN* KO blastocysts

Only *GFP* expressing cells with under the size of 20 μm and compact round shaped cells were chosen for SCNT. Before ET into recipient, KO efficiencies were check from cloned embryos. Eleven KO embryos were observed from total 37 embryos by T7E1 assay (KO efficiency rate; 30.0%) (Figure 27).

3.2. Production of *PRKN* KO piglets *via* SCNT

Among 14 recipients, 6 mothers (pregnancy rate; 42.9%) were pregnant at day 28 after ET with sonography. Four recipient (delivery rate; 28.6%) gave birth to 8 offspring those names were PDM1 to PDM8. PDM1 and PDM2 were delivered by C-sec from one mother, PDM3 to PDM6 were delivered by C-sec from another mother, PDM7 were delivered by C-sec from the third mother, and PDM8 were delivered by natural delivery from the last mother (Table 13). Among the 8 cloned piglets, PDM1 (live), PDM2 (euthanasia because of cleft), PDM4 (live), PDM7 (dead with unclear reason), and PDM8 (dead with unclear reason) were confirmed that normal piglets. Except them, 3 (PDM3; heterozygote; WT:A→G/-7, PDM5; homozygote; -4/+7, PDM6; homozygote; -78/-14) were confirmed that KO cloned piglets by sequencing analysis (Figure 28). The KO efficiency rate was 37.5% in this experiment. However these 3 KO pigs were all dead on

the day of birth (PDM3; unclear reason, PDM5; euthanasia because of cleft, and PDM6; stillbirth) (Table 14).

Even cell lines could not be established in PDM6 because of stillbirth, but PDM3 and PDM5 cell lines were cultured and cryopreserved, including cell lines from other wild type cloned piglets.

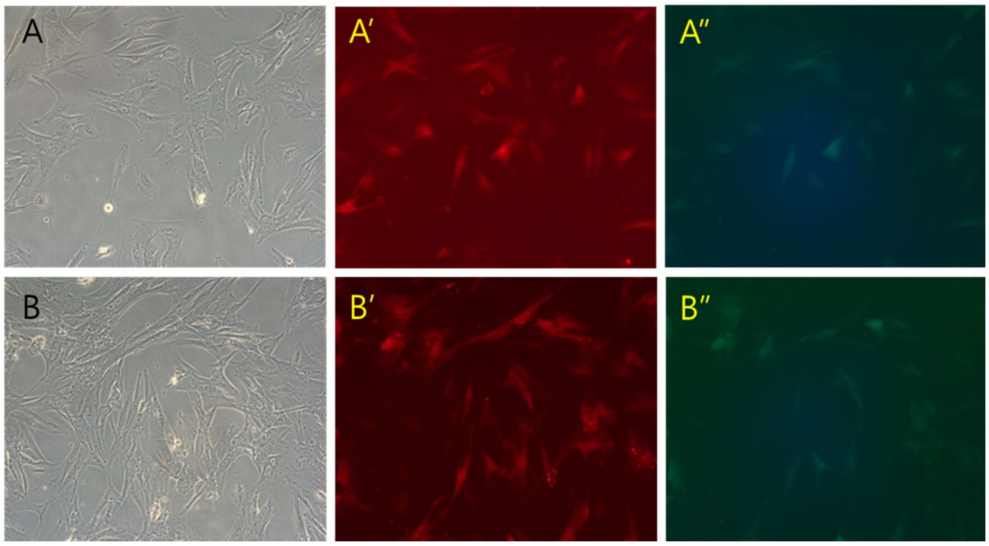


Figure 26. *PRKN* TALEN and reporter transfected cell lines. A, A', and A'' are pictures of randomly selected one site of TALEN and reporter transfected cell lines, and B, B', and B'' are pictures of another randomly selected one site of TALEN and reporter transfected cell lines with same electroporation conditions (Cell type; Sinclair male kidney, Cell number; 5×10^5 cells, Total amount of DNA; 5 μg (TALEN left: TALEN Right: Reporter = 2:2:1)). (A and B) TALEN and reporter transfected cell lines under the inverted microscope. (A' and B') Reporter gene inserted cells expressed RFP signals under the fluorescence microscope. (A'' and B'') GFP expressed cells were observed under the fluorescence microscope.

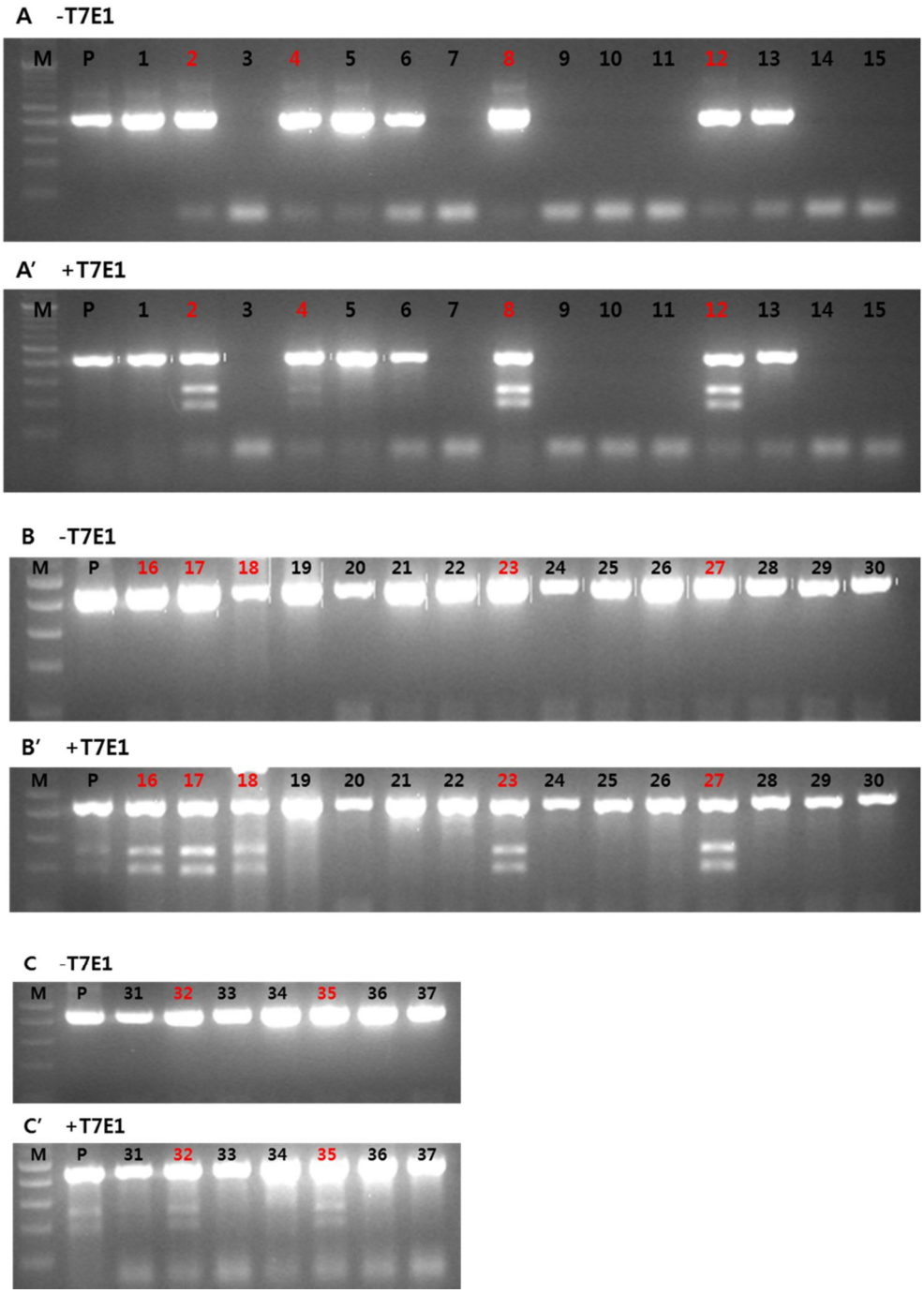


Figure 27. T7E1 assay result of SCNT derived embryos. Among 37 cloned embryos, 11 embryos (red colored) were confirmed as KO embryos by T7E1 assays (KO efficiency rate = 30%)

Figure 28. Sequencing results from PDM1 to PDM8. Compared to wild type, PDM1, PDM2, PDM4, PDM7, and PDM8 showed same sequences. PDM3 (heterozygote) showed 7 bps deletions in one allele, and A to G substitution was observed in another allele but wild type. PDM5 (homozygote) showed 4 bps deletions and 7 bps insertions in each alleles, and PDM6 (homozygote) showed 78 bps and 14 bps deletions in each alleles.

Table 13. Delivery methods and number of offspring for *PRKN* KO cloned pigs

No.	ID	Target gene	No. of offsprings	Delivery methods
1	Y27-4	*PRKN* KO	2	C-sec*
2	D4-3	*PRKN* KO	4	C-sec*
3	496	*PRKN* KO	1	C-sec*
4	494	*PRKN* KO	1	Natural delivery

*Caesarean section

Table 14. Current status of *PRKN* cloned piglets

ID	Modified genes	Sex	Fate	Cause of death
PDM1	None*	M**	Alive	-
PDM2	None*	M**	Dead	Cleft
PDM3	Hetero *PRKN* KO	M**	Dead	Unclear reason
PDM4	None*	M**	Alive	-
PDM5	Homo *PRKN* KO	M**	Dead	Cleft
PDM6	Homo *PRKN* KO	M**	Dead	Stillbirth
PDM7	None*	M**	Dead	Unclear reason
PDM8	None*	M**	Dead	Unclear reason

*None; Fail to KO *PRKN* gene

**Male

4. Discussion

I could produce *PRKN* KO pigs with highly selected TALEN pairs and reporters with no selection processes (e.g., antibiotic, fluorescent, and magnetic based selections.). After selection procedures, cells were much damaged (Jang *et al.*, 2006). These damaged cells were not appropriate for SCNT donors. Whole selection methods (e.g., antibiotic selection, fluorescence activated cell sorting (FACS), or magnetic activated cell sorting (MACS)) could damage to cells. MACS method gave the least damages to cells. However, in MACS, H-2K^k proteins were artificially introduced to the surface of cells (Kim *et al.*, 2013a), which hindered the fusion of oocyte cytoplasm to donor cell cytoplasm during SCNT (data not shown). Without selection processes, enrichment of KO cells were difficult. High performance TALEN pairs were selected to overcome this hurdle, and then knocked out cells were conveniently chosen under the UV microscope as donor cells for SCNT.

In conclusion, *PRKN* KO pigs were produced with SCNT combined with high performance TALEN without specific selection procedures. Three out of 8 pigs were confirmed as *PRKN* KO pigs by T7E1 and sequencing. Except stillbirth pig (PDM6), other *PRKN* KO pig cell lines were established and cryopreserved to further re-cloning experiment.

PART V

FINAL CONCLUSION

Firstly, *hTERT*s were randomly inserted pig cell line was established. These cell line was qualified by PCR based assay, karyotyping, sequencing, single cell colony formation assay, and telomerase activity test and so on. Finally I established exogenous gene overexpression cell line in pig.

CMAH and *GGTA1*, those were related with hyperacute rejection of pig organs to transplant into human body, were selected as KO targets. Each gene was knocked out separately. The knocked out cells were established and confirmed by sequencing, T7E1, fPCR, and FACS.

Nextly, these techniques, which worked properly in *in vitro*, were applied to *in vivo* transgenic pig production. Target genes, *SNCA* and *PRKN*, were selected to produce disease model pig. *SNCA* was well known gene for causative factor in PD. In contrast, *PRKN* was well known gene for protective factor in PD. Thus I scheduled to overexpress *SNCA* and *PRKN KO*. The *SNCA* overexpressed pigs were produced and confirmed by PCR, RT-PCR, ELISA, and motor scoring analysis. *PRKN* knocked out pigs were also delivered and confirmed by T7E1 and sequencing.

In conclusion, I demonstrated that immortalized porcine fibroblasts were successfully established using the human *hTERT* gene and the TALENs enabled gene disruptions (*CMAH* and *GGTA1*) in these immortalized cells *in vitro*. Additionally, these transfected cells also developed into the blastocyst stages *via* SCNT. Based on these *in vitro* studies, *SNCA* overexpressed pigs and *PRKN* KO pigs were successfully produced. The

live *SNCA* overexpressed pigs were determined as transgenic animals by PCR based analysis, however due to young age, only 1 year old pigs, clinical signs of PD should be intensively observed with time. Increasing concentrations of SNCA in blood and levels of motor scoring analysis in some pigs were expected positive signs of success. This needs additional follow up studies. In case of *PRKN* KO, no live piglets to date, further re-cloning plans are scheduled.

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국문초록

다양한 유전자 조절기술을 이용한 형질전환돼지의 생산

문 준 호

(지도교수: 장 구)

서울대학교 대학원

수의학과, 수의산과·생물공학 전공

현재까지 가장 이상적인 형질전환돼지를 생산하는 방법은 형질전환된 체세포를 핵이 제거된 난자에 주입하는 방법이다. 대부분의 형질전환돼지는 상대적으로 선별과정이 덜 복잡한 목적 유전자를 과발현하는 돼지이고 유전자 적중 방법으로 생산된 돼지의 수는 적다. 하지만 최근에 다양한 유전자 가위들 (즉, ZFN, TALEN, 또는 RGEN)이 각광을 받기 시작하면서 중대동물 형질전환 분야에서도 돼지를 포함한 다양한 종류의 유전자 적중된 동물들이 태어나기 시작했다.

돼지는 생물의학 연구에 있어서 중요한 동물모델이다. 하지만 배아줄기세포와 특성화된 세포주가 존재하지 않기 때문에

형질전환기술 개발과 형질전환동물 생산의 속도가 더디다. 본 연구에서는, 돼지의 특성화된 세포주가 존재하지 않는 점을 극복하기 위하여 *hTERT* 유전자를 과발현시킨 불멸화세포주를 확립하고 특성화하였다. 또한 이 세포를 이용하여 형질전환기술 즉, TALEN 을 통한 유전자 적중을 추가적으로 적용하였다. 이러한 유전자 과발현 기술과 유전자 적중 기술을 사용하여 각각의 형질전환돼지, 그 중에서도 신경질환돼지모델을 생산하였다.

본 연구에서는 돼지체세포를 불멸화하기 위해서 돼지 태아 섬유아세포를 분리하여 배양하였고 *hTERT* 를 전기적인 방법을 이용하여 세포 내로 도입하였다. Neomycin 을 이용하여 2 주간 형질전환된 세포를 선별하였고, 선별한 세포를 계대배양하였다.

목적 유전자가 돼지체세포에서 적절하게 발현하는지를 다양한 방법으로 확인하였다. 불멸화 돼지세포는 배가시간 (doubling time)이 대략 24 시간, 세포의 크기 역시 20 μm 이하로 최소 9 개월 이상 유지되는 것을 확인하였다. 하지만 같은 기간 동안 대조군 세포는 노화하여 더 이상 분열하지 못하는 것을 관찰하였다. 또한 불멸화 세포는 단 하나의 세포에서 100 mm 배양접시를 가득 채울 만큼 분열할 수 있는 능력이 있다는 것도 확인할 수 있었다.

이렇게 목적 유전자의 과발현 여부를 확인한 세포에 유전자 가위 중 하나인, TALEN 을 이용하여 *CMAH* 유전자를 적중시킨 세포주를 확립하였다. 개개의 colony 중에서 *CMAH* 유전자가 적중된 세포주를 확정하기 위해서 T7E1 를 진행하였고, fPCR 과 sequencing 을 통해서 그 중 3 개의 biallelic 유전자 적중 세포주를 확인하였다. *GGTA1* 유전자 적중도 위와 동일한 방법으로 진행하였다. 이렇게 만들어진 *hTERT* 유전자 과발현과 *CMAH* 유전자 적중이 동시에 된 세포주 중 하나를 이용해서 체세포 핵이식을 진행하였고, 여러 유전자가 조작된 세포주도 정상적으로 배반포까지 자라남을 증명하였다.

목적 유전자 과발현 및 적중된 형질전환돼지를 실제로 생산하기 위해서 파킨슨병과 관련된 유전자 중 *SNCA* 와 *PRKN* 을 각각 선별하였다.

먼저 *SNCA* 가 과발현하는 돼지 23 마리를 체세포 핵이식 기법으로 만들었다. 하지만 불명확한 이유로 현재는 4 마리만 살아있고, 그 4 마리 모두 형질전환동물임이 PCR 과 RT-PCR 을 통해 확인되었다. SNCA 단백질 발현 여부도 ELISA 를 이용해서 태어난 지 4 개월령에 실시하였을 당시 2 마리 (PDF4 와 PDF18)에서만 단백질 발현이 확인되었다. 하지만 단백질 발현의 정도는 1 년령이 되면서 변화하였고, PDF4 와

PDF20 은 SNCA 의 발현이 유의적으로 증가한 반면, PDF18 에서는 감소하였고, PDF16 은 변하지 않는 것을 확인하였다. 또한 행동학적 이상을 판단하기 위해 행동학적 분석을 대조군과 모든 형질전환돼지에서 5 개월령과 1 년령에 실시하였다 (대조군, 변화없음; PDF18, 감소; PDF4, PDF16, and PDF20, 증가; PD 유사행동을 보일 경우 수치가 증가). 그 결과 단백질 발현의 변화 유무와 행동학적인 이상 유무가 유사하게 나타나는 경향이 있다는 것을 확인하였다.

다음으로, 유전자 적중 돼지 생산을 위해 *PRKN* 을 선별하였다. *PRKN* 유전자를 자르는 TALEN 쌍을 리포터와 함께 돼지체세포에 주입하였고, 별다른 선별과정 없이 형질전환 2~3 일 후 초록색 형광빛을 발하는 세포만을 이용하여 체세포 핵이식을 진행하였다. 총 8 마리의 산자 (Parkinson’s disease male pig model; PDM1~8)가 태어났고, 이 중 3 마리 (PDM3; heterozygote, PDM5; homozygote, 그리고 PDM6; homozygote)가 T7E1 과 sequencing 을 통해서 최종적으로 확인되었다. 태어난 유전자 적중 돼지는 현재 모두 폐사한 상태이다 (PDM3; 사인불명, PDM5; 구개열로 안락사, 그리고 PDM6; 사산). 사산으로 태어나서 세포의 확립이 불가능했던 PDM6 를 제외한 나머지 2 마리의 유전자 적중 돼지에서

세포주를 확립하여 보관하고 있다. 현재 추가적으로 같은 유전자를 가진 돼지를 생산하기 위해 노력하고 있다.

결론적으로, 실험실 내에서 *hTERT* 유전자가 과발현된 불멸화 세포를 확립할 수 있었고, TALEN 을 이용하여 *CMAH* 유전자를 불멸화 세포에서 적중할 수 있었다. 추가적으로 이렇게 형질전환된 체세포도 체세포 핵이식에 공여세포로 사용이 가능함을 증명하였다. 이러한 방법적인 연구를 토대로, 실제로 *SNCA* 가 과발현하는 돼지와 *PRKN* 이 적중된 돼지를 생산하였다. 현재 살아있는 *SNCA* 과발현 돼지와 추후 재생산할 *PRKN* 적중돼지에 대해서는 추가적인 단백질 분석, 행동학적 분석, 그리고 뇌영상학적 분석등이 진행될 예정이다.

주요어: 형질전환동물, 체세포 핵이식, 유전자 과발현, 유전자 적중

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